Environmental regulation of the growth, physiology and virulence of *Legionella pneumophila*

Thesis

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ENVIRONMENTAL REGULATION OF THE GROWTH, PHYSIOLOGY AND VIRULENCE OF *LEGIONELLA PNEUMOPHILA*.

WILLIAM STUART MAUCHLINE

A thesis submitted in partial fulfilment of the requirements of the Open University for the Degree of Doctor of Philosophy.

The research presented in this thesis was undertaken at the Centre for Applied Microbiology and Research, Porton Down, Salisbury. Thames Water Utilities plc was the collaborating establishment.

December 1995

APPENDECIES NOT SCANNED AT THE REQUEST OF THE AWARDING UNIVERSITY

ABSTRACT

Members of the *Legionellaceae* cause respiratory infections in man; the most severe, pneumonic form is known as Legionnaires' disease. Of the 39 species described to date 16 have been associated with human disease, however the majority of reported cases of legionellosis are caused by *Legionella pneumophila* serogroup 1. A number of pathogenic bacteria regulate their virulence gene expression in response to environmental stimuli. Temperature and the availability of iron are considered to be stimuli which signal entry to a host environment.

The first part of this study utilised chemostat culture to investigate the influence of growth temperature and the availability of iron on the physiology, morphology and virulence of *L. pneumophila* serogroup 1. This study demonstrated, for the first time, that the virulence of *L. pneumophila* was significantly reduced ($P < 0.05$) when the culture temperature was lowered from 37 to 24°C and this modulation was reversed by returning the temperature to 37°C which resulted in a statistically significant ($P < 0.05$) increase in virulence. Further experiments demonstrated that the concentration of iron in the growth medium also had an effect on virulence. Contrary to expectations iron-limited cultures were less virulent than those grown iron-replete. This modulation was also reversible with a return to virulence when iron-replete conditions were restored.

The physiology and morphology of *L. pneumophila* were also influenced by both growth temperature and iron-limitation. At 24°C cultures consisted of flagellated short rods, whereas cultures grown at 37°C were pleomorphic and flagella were not evident. It was demonstrated that *L. pneumophila* accumulates the intracellular carbon storage compound, polyhydroxybutyrate, and that the proportion of the cell dry weight which it comprised varied with growth temperature, being maximal at 24°C. The ratio of saturated to unsaturated fatty acids in *L. pneumophila* decreased as the temperature was

reduced to 24°C; this is a common strategy designed to maintain membrane fluidity. Siderophore production was detected in iron-limited cultures but not in iron replete cultures. Protease production was also affected by both growth temperature and iron-limitation.

The BIOLOG bacterial identification system was modified for use with legionellae and this was used to investigate the metabolic versatility of these bacteria. A database containing substrate utilisation profiles of *Legionella* species was constructed using the modified system; this was then used to identify legionella isolates to species level.

Evaporative cooling towers are a significant source of Legionnaires' disease accounting for the majority of outbreak cases in the United Kingdom. In the second part of this study a microbiologically-contained, fully-functional evaporative cooling tower was constructed and used to investigate factors that could influence the growth of *L. pneumophila* in such systems. The mode of operation of the cooling tower was found to influence the multiplication of legionellae in the system. Low-usage situations resulted in enhanced growth of *L. pneumophila*. Growth of *L. pneumophila* demonstrated a significant positive correlation with water temperature but its concentration decreased with increased conductivity. The concentrations of calcium, magnesium, potassium and zinc and the total hardness of the water all exhibited inverse relationships with legionella population size. The protocol for the emergency disinfection of cooling systems recommended in the Report of the Department of Health Expert Advisory Committee on Biocides did not eradicate *L. pneumophila* from the experimental cooling tower.

DECLARATION

I declare that the research presented in this thesis is all my own work, except where otherwise indicated, and has not been submitted elsewhere for a research degree.

W. STUART MAUCHLINE

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. C.W. Keevil, Dr. R.B. Fitzgeorge and Dr. P.J.L. Dennis for their guidance and for proof reading this thesis. Thanks should also go to Mr. R Wait for mass spectrometry and nuclear magnetic resonance analyses, Mr. A.B. Dowsett for electron microscopy and Dr. R.B. Fitzgeorge for virulence determinations. I would also like to thank my colleagues, in particular Dr. A.A. West, for their support and encouragement.

Financial support was provided by the Medical Research Council (project grant G8823698SB) for the continuous culture study, and the Health and Safety Executive (reference 1/HPD/126/145/91) funded the cooling tower experiments.

Most of all I would like to thank my wife, Dr. M.L. Mauchline for her patience, understanding and support and without whom I would not have been able to complete this work.

PUBLICATIONS ARISING FROM THIS WORK

MAUCHLINE, W. S. & KEEVIL, C. W. (1991). Development of the BIOLOG substrate utilisation system for identification of *Legionella* spp. *Applied and Environmental Microbiology* **57**, 3345-3349.

MAUCHLINE, W. S., ARAUJO, R., WAIT, R., DOWSETT, A. B., DENNIS, P. J. & KEEVIL, C. W. (1992). Physiology and morphology of *Legionella pneumophila* in continuous culture at low oxygen concentration. *Journal of General Microbiology* **138**, 2371-2380.

MAUCHLINE, W. S., ARAUJO, R., FITZGEORGE, R. B., DENNIS, P. J. & KEEVIL, C. W. (1993). Environmental regulation of the virulence and physiology of *Legionella pneumophila*. In Legionella*: Current Status and Emerging Perspectives*, pp. 262-264. Edited by J. M. Barbaree, R. F. Breiman & A. P. Dufour. Washington, DC: American Society of Microbiology.

MAUCHLINE, W. S., JAMES, B. W., FITZGEORGE, R. B., DENNIS, P. J. & KEEVIL, C. W. (1994). Growth temperature reversibly modulates the virulence of *Legionella pneumophila*. *Infection and Immunity* **62**, 2995-2997.

JAMES, B. W., MAUCHLINE, W. S., FITZGEORGE, R. B., DENNIS, P. J. & KEEVIL, C. W. (1995). Influence of iron-limited continuous culture on the physiology and virulence of *Legionella pneumophila*. *Infection and Immunity* **63**, 4224-4230.

Reprints enclosed at rear cover.

DEDICATION

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CHAPTER 1: INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

Legionnaires' disease was recognised as a new form of pneumonia following a widely publicised outbreak of acute febrile respiratory illness among participants of the American Legion Convention in Philadelphia in 1976. Initially the epidemic was of unknown aetiology but the subsequent investigation led to the isolation of a new bacterium early in 1977, the Legionnaires' disease bacterium (Fraser *et al.*, 1977; Mcdade *et al.*, 1977). It was formally classified two years later as *Legionella pneumophila* in the family *Legionellaceae* (Brenner *et al.*, 1979).

1.2 CLASSIFICATION

1.2.1 Definition

The following definition of the *Legionellaceae* is based on that described by Brenner (1986). *Legionellaceae* are defined as Gram-negative, aerobic, nonspore-forming, nonacid-fast, unencapsulated rods of 0.3 to 0.9 μm in width and 2 to 20 μm or more in length. Elongated forms can be found in rich culture media (Pine *et al.*, 1979; Mauchline *et al.*, 1992). *Legionella* species are, with the exception of *L. nautarum, L. londiniensis* and *L. oakridgensis*, motile having one or more straight or curved polar or lateral flagella.

1.2.1 Biochemical profile

Legionella species utilise amino acids as their primary carbon and energy source and L-cysteine and iron salts are essential for growth *in vitro*. Carbohydrates are not appreciably catabolised. Legionellae are oxidase negative or weakly positive, nitrate-negative and urease negative and most species are able to liquefy gelatin. Branched chain fatty acids predominate in the cell wall, and they contain major

amounts of ubiquinones with more that 10 isoprene units in the side chain (Karr *et al.*, 1982; Moss *et al.*, 1983). The guanine plus cytosine (G+C) content of legionella DNA is 38 to 52 mol%. Inter species DNA relatedness is from 0 to 67%; however the DNA relatedness between strains within a species is 70% or usually higher (Brenner, 1986).

1.2.3 The family *Legionellaceae*

The family *Legionellaceae* consists of a single genus *Legionella*, although others, namely *Tatlockia* and *Fluoribacter* have been proposed (Garrity *et al.*, 1980). Although the genus is genetically heterogeneous it was considered to be a good and practical phenotypic genus (Brenner *et al.*, 1985). There are 39 species in the genus (Dennis *et al.*, 1993), 16 of which have been associated with human disease (Lee & West, 1991). *Legionella pneumophila,* the most common cause of disease, consists of 14 serogroups. *Legionella pneumophila* serogroup 1 accounts for the majority of clinical isolates (Reingold *et al.*, 1984) and strains within this serogroup associated with clinical cases share a common epitope, as indicated by monoclonal antibody typing (Watkins *et al.*, 1985). Strains of this microorganism isolated from clinical material react with MAB2 antibody, one of the monoclonal antibodies included in the international panel of monoclonal antibodies for subgrouping *L. pneumophila* (McKinney *et al.*, 1983).

Serogroup 1 can be divided into three major subgroups termed Pontiac, Bellingham and Olda, (Watkins *et al.*, 1985). Strains belonging to the Pontiac subgroup are isolated from around 88% of cases of Legionnaires' disease in Europe. In contrast, however, Pontiac strains are isolated infrequently from environmental sources not associated with outbreaks of disease (Watkins *et al.*, 1985; Brindle *et al.*, 1987).

1.3 CLINICAL ASPECTS AND EPIDEMIOLOGY

The clinical presentation varies widely with legionella infections, from asymptomatic infection, only demonstrable by sero-conversion (Dowling *et al.*, 1984), mild lower respiratory illness or to severe pneumonia-like symptoms. The principal forms of legionellosis are: Legionnaires' disease, an atypical pneumonia; Pontiac fever, a self-limiting influenza-like illness; and Lochgoilhead fever, an atypical Pontiac fever caused by *L. micdadei*.

1.3.1 Legionnaires' disease

Legionnaires' disease is essentially an acute lower respiratory tract infection which results from inhalation of Gram-negative bacteria belonging to the family *Legionellaceae* and their subsequent growth in the lung. It is an uncommon infection accounting for around 2% of community acquired pneumonias in the UK (Research Sub-Committee of the British Thoracic Association, 1987), with a total of 2480 cases in England and Wales reported to the Public Health Laboratory Service (PHLS) Communicable Disease Surveillance Centre (CDSC) in the period 1980 to 1993. The mean number of reported cases per year being 177 (Saunders *et al.*, 1994).

The mortality rate of Legionnaires' disease is between 10 to 20% and is comparable with that of other pneumonias. This figure may be higher in the case of nosocomially acquired infections (Bartlett *et al.*, 1986). The attack rate of the disease is around 1% with an incubation period of between 2 and 10 days. There is no evidence of person to person spread of the disease. The infective dose for man is unknown but is dependent on bacterial virulence and host susceptibility. Males are three times more likely to be infected than females and most cases occur in the 40 to 70 age group. Risk groups include smokers, alcoholics, diabetics and people with chronic illness or who are immunosuppressed.

Legionnaires' disease has no unique features in its clinical presentation. Fever occurs early in the onset of the disease and around 70% of patients have temperatures of 39.5°C or more. A dry cough may develop in about 75% of cases and the appearance of purulent sputum may occur after 3 to 4 days. Dyspnoea (laboured breathing) and respiratory distress are common and almost 80% of patients are under medical care within 7 days of the onset of the disease (Helms *et al.*, 1984). Non-respiratory symptoms, such as neurological symptoms, including confusion and delirium, and gastro-intestinal involvement (nausea, diarrhoea and vomiting) are seen in a number of cases.

1.3.2 Pontiac fever

Pontiac fever derives its name from an epidemic of acute febrile illness which occurred in 1968 at a county health department facility in Pontiac, Michigan (Glick *et al.*, 1978). (It was not until some ten years later that *L. pneumophila* was implicated as the aetiological agent.) It is a self-limiting influenza-like condition, associated with the inhalation of legionellae, which resolves spontaneously after 2 to 5 days. The incubation period is short (36 to 48 hours) and the attack rate is high (95%).

1.3.3 Lochgoilhead fever

During the New Year holiday period (1987-88) there was an outbreak of a Pontiac fever-like illness at a leisure complex at Lochgoilhead, on the west coast of Scotland (Goldberg *et al.*, 1989). Of the 187 visitors to the centre, for whom case histories were obtained, 170 (91%) had symptoms; the age range was 2 to 72 years; and some of the clinical features were atypical of Pontiac fever, in that breathlessness and long-lasting illness were reported.

1.4 PATHOGENESIS

Legionella pneumophila is an intracellular pathogen of the mononuclear phagocyte, principally monocytes and aleveolar macrophages (Horwitz & Silverstein, 1980; Nash *et al.*, 1984) Phagocytosis by human monocytes is mediated by a three-component phagocytic system consisting of monocyte complement receptors, CR1 and CR3, fragments of complement component C3 and the major outer membrane protein (MOMP) on the surface of *L. pneumophila.* C3 attaches selectively to MOMP by the alternative pathway of complement activation (Payne & Horwitz, 1987; Bellinger-Kawahara & Horwitz, 1990; Marra *et al.*, 1990). *Legionella pneumophila* is phagocytised by host cells, usually by coiling phagocytosis, in which long phagocyte pseudopods coil around the bacterium as it is internalised, and resides in a specialised phagosome which does not fuse with lysosomes or become highly acidified (Horwitz, 1983b, c, 1984; Horwitz & Maxfield, 1984). A mutant of *L. pneumophila* which does not inhibit phagosome-lysosome fusion was shown to be avirulent for monocytes (Horwitz, 1987).

1.4.1 Host Immunity

The host defends itself against *L. pneumophila* by cell-mediated immune mechanisms. Activated human monocytes and alveolar macrophages have been shown to inhibit intracellular multiplication of the pathogen (Horwitz & Silverstein, 1981; Nash *et al.*, 1984, 1988; Bhardwaj *et al.*, 1986). Polymorphonuclear leukocytes (PMN) activated by interferon-gamma and tumour necrosis factor have been found to have an enhanced, although modest, ability to kill *L. pneumophila* (Blanchard *et al.*, 1989).

Down-regulation of complement receptors on activated mononuclear phagocytes by interferon-gamma results in a 50% reduction in the number of *L. pneumophila* cells internalised which in turn reduces access to the intracellular environment required for multiplication of the pathogen (Horwitz & Silverstein, 1981; Payne & Horwitz, 1987).

In addition, activated macrophages and monocytes slow the multiplication rate of the bacteria which do get internalised. Interferon gamma-activated monocytes do this by reducing the intracellular availability of iron through down regulation of transferrin receptor expression and intracellular ferritin concentration (Byrd & Horwitz, 1989, 1990, 1993).

Studies in PMN-deplete guinea pigs have indicated these defence cells play an important role in host immunological response to infection with *L. pneumophila* (Fitzgeorge *et al.*, 1988). Apolactoferrin has been shown to inhibit the intracellular growth of *L. pneumophila* in human monocytes and is the major protein in the specific granules that PMN release at the sites of infection. Thus PMN may also cooperate with monocytes in host defence by providing infected mononuclear phagocytes with this iron binding protein (Byrd & Horwitz, 1991).

1.4.2 Virulence

The macrophage infectivity potentiator (Mip) protein is the only molecule in *L. pneumophila* that has been proved to be a virulence determinant. This 24 kDa surface protein is required for the full expression of virulence in both mononuclear phagocytes and guinea pigs (Cianciotto *et al.*, 1989, 1990). Mip has also been shown to inhibit protein kinase C activity (Hurley *et al.*, 1993). The *mip* gene which codes for the Mip protein is common to the whole genus; in addition it is now known that this gene is conserved in both prokaryotes and eukaryotes, including humans (Engleberg, 1993). There are a number of other molecules which are of potential importance in the pathogenesis of *L. pneumophila*. The major cytoplasmic membrane protein (MCMP) which is a genus common antigen, can elicit protective immunity (Gabay & Horwitz, 1985a). MCMP is a heat shock protein, Hsp60, the synthesis of which is rapidly increased by virulent, but not avirulent, *L. pneumophila* on becoming internalised by macrophages (Hoffman *et al.*, 1993). In addition it is moved from the cytoplasm and periplasm to the cell surface where it possibly interferes with phagosome membrane

proteins involved in phagolysosomal fusion (Hoffman *et al.*, 1993). The major outer membrane protein (MOMP) is a cation selective porin (Gabay & Horwitz, 1985b) and also acts as the site of attachment for complement component C3 (Bellinger-Kawahara & Horwitz, 1990). Phospholipase C which hydrolyses phosphatidylcholine may also have a role in virulence (Baine, 1988). Although the peptidoglycan-associated lipoprotein may not have an active role in virulence it reliably induces a humoral response in guinea pigs (Engleberg, 1993). The major secretory protein (MSP) of *L. pneumophila* is a zinc metalloprotease with weak haemolytic activity which is produced intracellularly in monocytes (Dreyfus & Iglewski, 1986; Keen & Hoffman, 1989; Clemens & Horwitz, 1990; Williams *et al.*, 1993). This protease is structurally and functionally homologous to *Pseudomonas aeruginosa* elastase (Black *et al.*, 1989). MSP, although it is a highly potent immunoprotective molecule, does not appear to be a virulence determinant in guinea pigs (Blander *et al.*, 1990) or in human mononuclear phagocytes (Szeto & Shuman, 1990); however it probably does contribute to the pathogenesis of the disease (Williams *et al.*, 1993).

It is now becoming evident that many different genes are required for the full expression of *L. pneumophila* virulence. *Legionella pneumophila* responds to the intracellular environment of the macrophage by altering gene expression. During infection of macrophage-like cell line, U937, the expression of at least 35 proteins was induced and one of these proteins was not detected in bacteria grown in a defined culture medium (Abu Kwaik *et al.*, 1993a). The expression of at least 32 proteins were selectively repressed during intracellular growth, and 9 were undetectable. The expression of 19 of these macrophage-induced proteins (MINs) could be induced *in vitro* by exposure to stress conditions such as heat shock, exposure to H_2O_2 and osmotic shock (Abu Kwaik *et al.*, 1993a). Iron chelation, anaerobiosis and acid shock also induced some of the MINs (Abu Kwaik *et al.*, 1993b). Preferential expression of specific proteins by virulent *L. pneumophila* internalised by L929 cells was reported by (Hoffman *et al.*, 1993). Although both virulent and avirulent cells were phagocytised,

only virulent bacteria prevented phagosome-lysosome fusion. The virulent cells preferentially synthesised Hsp60, whereas the avirulent cells appeared to preferentially express low-molecular-weight proteins. In addition to this the virulent strain synthesises much less MOMP than the avirulent one (Hoffman *et al.*, 1993).

1.4.3 Transmission

The most important route of infection is inhalation of an aerosol containing *L. pneumophila*, or another pathogenic *Legionella* species (Fraser, 1980; Bartlett *et al.*, 1986). A guinea-pig model for aerosol transmission of Legionnaires' disease was developed by Baskerville *et al.* (1981). The pneumonic lesions produced by aerosol infection of guinea-pigs were very similar to those in the human disease. Using this model Fitzgeorge *et al.* (1983) demonstrated that administration of *L. pneumophila* as a small particle aerosol ($< 5\ \mu m$) resulted in a fatal infection with a dose of 10^3 bacteria, whereas a challenge of 10^9 bacteria of same strain was not lethal when delivered by the intranasal route. They concluded that particle size was of crucial importance in the establishment of infection, with particles of $< 5\ \mu m$ able to penetrate to and be deposited in alveoli and terminal bronchioles, whereas intranasal instillation would generate very few particles small enough reach these sites. Although intraperitoneal infection with *L. pneumophila* causes mortality in guinea-pigs, it is less virulent by this route than by aerosol infection (Berendt *et al.*, 1980; Fitzgeorge *et al.*, 1983). Furthermore, Fitzgeorge *et al.* (1983) found that aerosol infection was capable of differentiating between the virulence of *L. pneumophila* strains whereas the median lethal dose for intraperitoneal infection was similar for all the strains they tested.

The above model of legionella infection was used in the current investigation of environmental regulation of the virulence of *L. pneumophila* as it uses what is widely regarded as the natural route of infection and allows a quantitative assessment of virulence.

1.5 PHYSIOLOGY

1.5.1 Nutrition

The primary sources of carbon and energy for legionellae are amino acids (Pine *et al.*, 1979; George *et al.*, 1980; Ristroph *et al.*, 1981; Tesh & Miller, 1981). Indeed legionellae have been successfully cultured using defined medium composed of amino acids (Pine *et al.*, 1979, 1986b; George *et al.*, 1980; Ristroph *et al.*, 1981; Tesh & Miller, 1981; Mauchline *et al.*, 1992). The precise amino acid requirements of legionellae are subject to some disagreement but the consensus is that arginine, cysteine, isoleucine, leucine, methionine, serine, threonine and valine are required (Hoffman, 1984). Phenylalanine and tyrosine were found to be necessary for growth in 10 strains of *L. pneumophila* by George *et al.* (1980), and that these amino acids could substitute for each other. The easily metabolised amino acid glutamate, although not required in the presence of the other twenty amino acids, can act as an energy source for legionellae (Weiss *et al.*, 1980; Tesh & Miller, 1981). Serine, threonine, histidine, tryptophan and tyrosine were found to serve as energy sources (Pine *et al.*, 1979). Only serine and threonine were found to be major energy sources for *L. pneumophila* (George *et al.*, 1980) but based on stimulation of oxygen uptake (Tesh *et al.*, 1983) demonstrated that glutamate and tyrosine could also be utilised as energy sources. It is likely that some of the disagreement on which amino acids are required arises from the differing culture conditions and growth media used in the various studies.

Although early reports suggested that *L. pneumophila* is capable of synthesising all its requirements for vitamins and vitamin-like compounds (Ristroph *et al.*, 1980; Ristroph *et al.*, 1981). Pine *et al.* (1986a) reported that guanine was required for the growth of some non-*L. pneumophila Legionella* species and that other purines, pyrimidines or nucleosides could also stimulate growth.

The trace metal requirements of *L. pneumophila* are calcium, copper, cobalt, iron,

magnesium, manganese, molybdenum, nickel, vanadium, zinc (Reeves *et al.*, 1981), and potassium (Tesh & Miller, 1982).

1.5.2 Metabolism

Glucose is slowly oxidised by *L. pneumophila*; however a high proportion of its carbon is incorporated into cell components (Weiss *et al.*, 1980). Radiorespirometric studies by Tesh *et al.* (1983) confirmed the earlier assertions of Weiss *et al.* (1980) that glucose was metabolised by the pentose phosphate and the Entner-Doudoroff (ED) pathway rather than the Embden-Meyerhoff-Parnas (EMP) pathway. However, Keen and Hoffman (1984) found no evidence of the presence of key enzyme activities belonging to the ED pathway, but they did find enzyme activities consistent with the pentose cycle. These researchers reported the presence of a number of catabolic enzyme activities of EMP pathway, including hexokinase, phosphoglucose isomerase, phosphofructokinase and fructose-1,6-biphosphate aldolase which were low compared to the activities present in *Escherichia coli*. The specific activity of the anabolic enzyme fructose-1,6-bisphosphate was some ten times greater than that of phosphofructokinase, which was concluded by the authors to be strongly indicative that the direction of the EMP pathway was towards gluconeogenesis. In addition this study found no evidence for active uptake of glucose by *L. pneumophila*, however glutamate uptake could be inhibited by a decoupling agent (Keen & Hoffman, 1984).

The Krebs cycle is complete in *L. pneumophila* with the activity of the glyoxylate bypass being absent or only present at a very low level (Hoffman, 1984). It would appear that the Krebs cycle is the primary route of energy production and carbon assimilation in this organism (Hoffman & Pine, 1982). Serine enters the Krebs cycle after it has been deaminated to pyruvate by L-serine dehydratase (dehydration of serine followed by a spontaneous deamination), with the concomitant release of ammonia (Keen & Hoffman, 1984). It was proposed that glutamate is metabolised after being deaminated to α-ketoglutarate in a reaction catalysed by

glutamate-aspartate transaminase (Keen & Hoffman, 1984).

1.5.3 Respiratory metabolism

The electron transport chain of *L. pneumophila* is composed of cytochromes of the *c, b, a* and *d* types (Kronick & Gilpin, 1980; Hoffman & Pine, 1982). It is likely that the respiratory chain is branched and is terminated by several oxidases. Legionellae are relatively resistant to cyanide but highly sensitive to the respiratory inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide (Hoffman & Pine, 1982). The major quinone is ubiquinone which has a terpene side chain containing 10 to 14 carbon atoms which is unique to legionellae as other bacteria have carbon chain lengths of less than 10 carbons (Karr *et al.*, 1982).

Legionellae, in common with a number of pathogenic bacteria, such as *Neisseria gonorrhoea* (Norrod & Morse, 1982), *Spirillum volutans* (Padgett *et al.*, 1982) and *Campylobacter jejuni* (Hoffman *et al.*, 1979), are sensitive to the presence of low levels of hydrogen peroxide. Superoxide and hydrogen peroxide radicals are generated by the autoxidation of media constituents. Exposure of yeast extract (YE) broth to fluorescent light generates 3 μM h^{-1} superoxide free radicals and 16 μM h^{-1} hydrogen peroxide. Various *Legionella* species have been shown to be killed by concentrations of 25 μM or greater for 1 hour of hydrogen peroxide (Locksely *et al.*, 1982; Hoffman *et al.*, 1983). Photochemical oxidation of YE broth leads to the production of 50 μM hydrogen peroxide in three hours. The inclusion of charcoal in culture media reduces these toxic effects: activated charcoal prevents photochemical oxidation of YE medium, prevents the auto-oxidation of cysteine and decomposes hydrogen peroxide and superoxide radicals (Hoffman *et al.*, 1983). The presence of cysteine and ferric iron, both essential components of legionella culture media, is known to lead to the formation of hydrogen peroxide and superoxide radicals (Nyberg *et al.*, 1979; Norrod & Morse, 1982). The enzyme activities which are protective against oxidative damage, catalase, peroxidase and superoxide dismutase (SOD), are not uniformly present in all *Legionella*

species: although all species express SOD activity to some extent they do not all express the other two activities, in fact the *L. pneumophila* strains investigated appear to have only peroxidase (Pine *et al.*, 1986b).

Legionellae are microaerophilic in nature: oxygen is required for respiration but they are very sensitive to reactive oxygen derivatives; their growth is enhanced under microaerobic conditions.

1.5.4 Cysteine auxotrophy

Legionellae require cysteine for growth but unlike other cysteine auxotrophs (*e.g. E. coli*, salmonellae and bacilli) this requirement cannot be satisfied by cystine; however in the case of *L. pneumophila* glutathione can act as a substitute. Cysteine is rapidly oxidised in both YE medium and in ACES-buffered chemically defined (ABCD) broth, where including the presence of charcoal does not prevent the auto-oxidation. In YE broth it oxidises at the rate of 18 to 28 μM min^{-1} levelling off at around 0.5 μM (Hoffman, 1984), and in basal ABCD broth (*i.e.* deficient in pyruvate and α-ketoglutarate) the oxidation rate is approximately 8 μM min^{-1} even when light is excluded; the final concentration of cysteine is 0.12 mM in the absence of light or slightly lower when illuminated (Pine *et al.*, 1986b). Ferric pyrophosphate is reduced at the rate of 9 μM min^{-1} in YE medium during the oxidation of cysteine. Since cysteine is unstable in media it is usually included in excess (3.6 mM in ABCD broth) to ensure growth of legionellae. Cysteine is taken up by *L. pneumophila* at a rate of 2.5 pmol min^{-1} $mg_{(dry\ weight)}^{-1}$, about one quarter the rate of uptake for *E. coli*, and incorporated into proteins. *Legionella pneumophila* lacks serine transacetylase and *o*-acetylserine sulphydrylase which are two key enzymes for the biosynthesis of cysteine. In contrast *L. oakridgensis*, the only *Legionella* species not auxotrophic for cysteine, possesses both these enzymes and they appear to be constitutively expressed (Hoffman, 1984).

1.6 IRON

Iron is required for a number of cellular functions and accordingly is an essential nutrient for the majority of bacteria. Iron is perhaps the most versatile of all biocatalytic elements: it can undergo a reversible change in its redox state involving only one electron; the redox potentials of enzymes containing iron spans a range of almost 1000 mV. Metalloproteins which contain iron fall into three main categories. Firstly, proteins which form complexes with iron, such as the iron-binding glycoproteins found in the bloodstreams of vertebrates, transferrin and lactoferrin; ovotransferrin a related protein from egg white; and the iron storage proteins ferritin and bacterioferritin. The second category comprises proteins which bind oxygen reversibly, such as haemoglobin and leghaemoglobin. The final category contains those proteins which function as enzymes concerned primarily with redox reactions: electron transfer enzymes, haem containing (*e.g.* cytochromes) and non-haem type (*e.g.* iron-sulphur proteins); flavoproteins such as NADH dehydrogenase; and other iron containing enzymes.

Although iron is the fourth most abundant element its biological availability in the environment is severely limited. In aqueous environments spontaneous oxidation leads to the formation of insoluble ferric hydroxides and oxyhydroxides at neutral or alkaline pH (Griffiths, 1987a).

1.6.1 Iron and infection

The availability of free iron *in vivo* is severely limited: it is found intracellularly, in ferritin, haemosiderin or haem, and that which is extracellular is bound to high affinity iron-binding proteins (transferrin and lactoferrin). The amount of free iron in equilibrium with iron binding proteins is of the order of 10^{-18} M (Bullen *et al.*, 1978). It has been reported that Gram-negative bacteria require between 0.3 to 1.8 μM iron for growth (Weinberg, 1974). Accordingly pathogenic bacteria have evolved a number of strategies for assimilating iron in animal hosts. Iron can be obtained directly from

the host's iron-binding glycoproteins or through proteolytic cleavage of these proteins; by reduction of Fe^{3+} complex to Fe^{2+} complex resulting in the release of Fe^{2+}; by direct binding of bacterial surface receptors with iron bound to glycoprotein; by the production of low molecular mass iron-binding compounds, known as siderophores, capable of removing iron from serum proteins (Lankford, 1973; Neilands, 1981, 1984b).

1.6.2 Legionellae and iron

Iron is required for the growth of *L. pneumophila* (Feeley *et al.*, 1978), and Reeves *et al.* (1981) found that a concentration of 20 μM iron was required for maximum growth of *L. pneumophila* Philadelphia 1 in their defined medium. Iron incorporated into *L. pneumophila* is found in seven major iron-containing proteins including iron superoxide dismutase (Mengaud *et al.*, 1993). The major iron containing protein has an apparent molecular mass of 210 kDa under non-denaturing conditions and 85 to 90 kDa under denaturing conditions (Mengaud *et al.*, 1993).

Mononuclear phagocytes, like other vertebrate cells, acquire iron via transferrin receptors on their surface. The transferrin receptor is comprised of two 93 kDa disulphide-linked proteins, each capable of binding a diferric transferrin molecule at neutral pH: their expression is regulated at the transcriptional level according to the demand of the cell for iron (Rao *et al.*, 1986). Uptake occurs by endocytosis of multiple transferrin-receptor complexes within a clathrin-coated vesicle. This is followed by the acidification of the vacuole resulting in the reduction and release of iron from the complex, the iron is transported to the cytoplasm, and the apotransferrin-receptor complex is returned to the cytoplasmic membrane (Nunez *et al.*, 1990). Apotransferrin is released since it has a lower affinity for the receptor than iron-transferrin (Young, *et al.*, 1984). Intracellularly *L. pneumophila* obtains iron from the intermediate labile iron pool of the monocyte (Byrd & Horwitz, 1989). This iron is derived from iron-transferrin or iron-lactoferrin which enters via specific transferrin or

lactoferrin receptors on the surface of the monocyte, and from the iron storage protein ferritin (Byrd & Horwitz, 1989, 1990, 1991). Gebran *et al.* (1994) reported that the ability of *L. pneumophila* to grow in elicited macrophages and not in resident macrophages of A/J mice was due, at least in part, to the intracellular availability of iron. They showed that elicited A/J mouse macrophages, which allow intracellular multiplication of *L. pneumophila*, expressed more transferrin receptors and had a higher intracellular iron content than resident, non-permissive macrophages, and that multiplication could be inhibited by chelating iron from the intracellular labile pool or by blocking the transferrin receptors. In addition, and by way of emphasising the requirement for iron, normally non-permissive resident macrophages could support intracellular growth in the presence of iron salts (Gebran *et al.*, 1994).

Iron-restriction appears to have a role to play in the host's immunological response to infection with *L. pneumophila*. Host iron-binding proteins have been shown to be antagonistic to the growth of legionellae. Lactoferrin, which is found in mucosal secretions, in specific granules in polymorphonuclear leukocytes and which is internalised via specific receptors on monocytes, inhibits or promotes *L. pneumophila* intracellular multiplication in human mononuclear phagocytes depending on its degree of iron-saturation (Byrd & Horwitz, 1991). Similarly, human apolactoferrin is bactericidal for *L. pneumophila* whereas iron-saturated lactoferrin has no activity (Bortner *et al.*, 1986). In addition, apotransferrin is bactericidal to *L. pneumophila* (Quinn & Weinberg, 1988). Earlier, Muller *et al.* (1983) showed that, following aerosol infection with *L. pneumophila*, the serum iron level in guinea pigs was significantly reduced and this coincided with an increase in the concentration of serum transferrin. Intracellular growth of *L. pneumophila* is inhibited by human monocytes and alveolar macrophages activated by cytokines or recombinant human interferon gamma (Horwitz & Silverstein, 1981; Horwitz, 1983a; Bhardwaj *et al.*, 1986; Nash *et al.*, 1988). This antimicrobial activity is the result of restricting the availability of intracellular iron (Byrd & Horwitz, 1989).

There is some disagreement as to the mechanism(s) by which legionellae obtain iron. There are conflicting reports on the production of siderophores by *Legionella* species. Reeves *et al.* (1983) failed to detect the production of the common types of siderophores, namely hydroxymate or phenolate siderophores, by several *Legionella* species during iron-restricted growth. However, using different detection methods Goldoni *et al.* (1991) reported the production of siderophore-like activity by eight serogroups of *L. pneumophila* and several other *Legionella* species when grown under iron-restricted conditions. The former study employed chemical assays which recognise these specific classes of siderophores and a bioassay which also specifically detects the presence of either hydroxymate or phenolate siderophores, whereas the latter employed the universal siderophore assay of Schwyn and Neilands (1987) which directly detects iron sequestering activity. The authors of the second report also failed to find either hydroxymate or catechol (a type of phenolate siderophore) siderophores in *Legionella* species. Warren and Miller (1980) also detected production of siderophores by *L. pneumophila* using a human serum diffusion assay and suggested that on the basis of analysis by thin layer chromatography that the siderophores were of catechol structure, however little experimental detail was published. Since there are several siderophores known to be neither hydroxymates or catechols (Neilands, 1984a; Schwyn & Neilands, 1987) the apparent conflict is perhaps attributable to the assay methods used in each study.

Johnson *et al.* (1991) compared the iron requirement and uptake of a virulent and an avirulent strain and found that the former required 3 μM iron for growth whereas the avirulent strain needed 13 μM iron. They reported that neither strain could obtain iron from transferrin but that both strains possessed an iron reductase (ferric citrate reductase). The reductase of virulent cells showed greatest activity when NADH was the reductant, while avirulent cells had maximum activity with NADPH as reductant. During phagocytosis there is an eight-fold increase in NADPH oxidase activity, in contrast the concentration of NADH remains constant (Rossi *et al.*, 1972). This led

Johnson *et al.* (1991) to suggest that the reduction in the concentration of NADPH may restrict the ability of the avirulent strain to obtain iron intracellularly. They also advanced the idea that the avirulent strain's higher demand for iron may contribute to its inability to survive in phagocytic cells.

In contrast to many other Gram-negative bacteria which produce high molecular weight iron-regulated outer membrane proteins in response to iron-restriction (Brown & Williams, 1985), iron-deplete conditions did not result in the expression of a such high efficency uptake system in *L. pneumophila* (Barker *et al.*, 1993).

1.7 BACTERIAL REGULATORY SYSTEMS

Most pathogenic bacteria spend at least some of their time external to their host, and so have to adapt to different environments. Expression of virulence factors in external environments is unnecessary, and may put the bacterium at a selective disadvantage. It is, therefore, advantageous for bacteria to exercise the same level of control over the expression of virulence genes, as over genes encoding metabolic pathways. Genetic control of virulence can be divided into two categories: random and non-random. Expression of virulence factors in *Bordetella pertussis* is subject to two forms of genetic control; phase variation and antigenic/phenotypic modulation, representing both random and non-random control, respectively (Lacey, 1960; DiRita & Mekalanos, 1989).

1.7.1 Random control

Random forms of control include phase or antigenic variation, where a fraction of a given population express a different phenotype from the rest of the population. This form of regulation usually involves surface structures such as flagella, pili, outer membrane proteins and capsules which impart some advantage in colonising the host environment, such as enhanced attachment to host tissue or enabling the microorganism to avoid phagocytosis. Since surface structures are major targets of the host antibody

response the ability to rapidly vary these surface features allows a portion of the bacterial population to avoid clearance by the immune system. Alternatively the expression of a variety of phenotypes with differing adhesion factors enhances the likelihood of finding suitable receptors on the wide variety of tissues presented by the host. Most strains of *E. coli* produce type 1 pili (or fimbriae), cells expressing such pili adhere to mannose containing molecules on a number of types of eukaryotic cells. Adherent Fim^+ bacteria compete better for nutrients than do Fim^- strains, however those with the Fim^+ phenotype are more susceptible to phagocytosis and clearance by the host immune system. Type 1 producing *E. coli* alternates between the Fim^+ and the Fim^- states and it has been shown that this phase variation is under transcriptional control and occurs at a rate of around one cell per 1000 generations (Eisenstein, 1981).

1.7.2 Non-random

Phenotypic modulation of gene expression is the process in which the entire bacterial population alters its expression of one or more genes in response to an environmental stimulus (or combination of stimuli). This non-random regulation requires the pathogenic bacterium to sense signals in the environment and to regulate virulence gene expression accordingly and is of particular importance in adapting the pathogen as it moves from one environmental niche to another. Coordinate regulation of virulence genes has been reported in a number of pathogens, including *Shigella* spp. (Maurelli *et al.*, 1984), *Yersinia* spp. (Straley & Bowmer, 1986), *Bordetella pertussis* (Lacey, 1960), *Listeria monocytogenes* (Leimeister-Wachter *et al.*, 1992) and *Staphylococcus aureus* (Rescei *et al.*, 1986). These bacteria respond to specific environmental stimuli such as temperature, pH, concentration of specific ions, osmolarity or to some combination of these factors (Maurelli, 1989; Miller *et al.*, 1989). The subject of signal transduction and virulence regulation has recently been reviewed by Gross (1993).

Utilising "signature" molecules which are specifically produced by host tissues is

probably the most effective way for a bacterium to sense that it is in a host. This is the mechanism used by two phytopathogens, *Agrobacterium tumefaciens* (Binns & Thomashow, 1988; Ankenbauer & Nester, 1990) and *Pseudomonas syringae* (Mo & Gross, 1991). However, this mechanism has yet to be recognised in human pathogens. The next best means of control of virulence is through use of environmental signals as prompts. Indeed, many pathogenic bacteria use environmental cues that signal entry into host tissue. The two parameters which have been most intensively studied are elevated temperature and low iron concentration. Given the life style of legionellae there would seem to be a reasonable prima-facie case for considering temperature and iron-restriction as possible regulatory stimuli. An investigation of the influence which these environmental signals, particularly temperature, have upon the growth, physiology and virulence of *L. pneumophila* comprises a major part this thesis.

1.7.3 Temperature regulation of virulence

Before discussing temperature as an environmental stimulus of gene expression it is important to draw the distinction between thermoregulation of gene expression and the "heat shock response". Temperature regulation of gene expression may be distinguished from the "heat shock response" in two ways. Firstly, it involves genes not necessarily transcribed from classic heat shock promoters and secondly, these genes are induced specifically by temperature changes and not by the numerous other environmental stimuli which have been shown to activate the heat shock regulon members. Thermoregulated genes have promoters recognised by σ^{70} and their thermoresponsiveness is determined by *trans*-acting transcription factors which can be highly specific or, in some cases, highly pleiotropic. Many of the best understood examples of thermoregulation involve virulence genes under the positive control of members of the AraC family of transcription factors (These are regulatory proteins named after the prototypic member AraC, the L-arabinose operon regulator; they are associated either with regulation of carbohydrate metabolism or virulence). Several of these genes are negatively regulated by the nucleoid-associated protein, H-NS

(Dorman, 1994).

Temperature is a good candidate for a potential environmental stimulus, since the constant internal temperature of mammals is for the most part higher than that of the external environment, indeed growth temperature has been shown to affect the virulence of a number of bacterial pathogens (Maurelli, 1989).

Shigellae are Gram-negative enteric pathogens which cause shigellosis and bacillary dysentery. The disease is characterised by the ability of the bacteria to penetrate colonic epithelial cells, multiply intracellularly and spread to adjacent cells. It has been demonstrated by Maurelli *et al.* (1984) that the genes implicated in the primary step of *Shigella* pathogenesis are temperature regulated. When grown at 37°C, strains of *Shigella dysenteriae, S. flexneri* and *S. sonnei* are able to invade mammalian cell, however this ability is blocked when these bacteria are grown at 30°C. Enteroinvasive *E. coli* (EIEC) are similar to *Shigella* spp. both biochemically and in their pathogenesis. Expression of virulence in strains of EIEC are also temperature regulated (Small & Falkow, 1988).

A more complicated two-component virulence regulatory system based on the availability of calcium as well as temperature occurs in members of the genus *Yersinia*. Yersiniae cause a number of human infections ranging from bubonic plague (*Y. pestis*) to acute gastroenteritis (*Y. enterocolitica*) and mesenteric adentis (*Y. pseudotuberculosis*). All three species exhibit tropism for lymphoid tissue and an intracellular stage in the disease cycle (Cornelis *et al.*, 1987). In addition, these species all possess plasmids of around 70 kb which are necessary for virulence and are structurally and functionally homologous (Portnoy & Martinez, 1985). These plasmids are associated with a number of temperature regulated phenotypes including: production of V and W antigens (Perry & Brubaker, 1983), autoagglutination (Laird & Cavanaugh, 1980), serum resistance (Pai & DeStephano, 1982), cytotoxicity for

macrophages (Goguen *et al.*, 1986), the expression of certain *Yersinia* outer membrane proteins (Yops; Bolin *et al.*, 1985, 1988) and the low calcium response (Higuchi & Smith, 1961; Brubaker, 1983). The low calcium response (LCR) is manifested as restriction of growth and production of Yops when virulent yersiniae are shifted from 26°C to 37°C in the absence of calcium (Brubaker, 1983). This represents two distinct regulatory networks, neither of which is fully understood. However, there is clear evidence that transcription of the Yop regulon is positively regulated by the VirF protein in response to an increase in temperature, and negatively regulated by calcium by a VirF-independent mechanism. Furthermore, transcription of the plasmid encoded *yop*, *yadA* and *vir* genes is thermoregulated (Cornelis, 1992). The regulation of chromosome-encoded virulence factors of yersiniae is counter-intuitive, with expression being optimal at low temperatures. *Yersinia enterocolitica* heat-stable toxin (Yst) is only detected in supernatants of cultures grown at less than 30°C (Cornelis, 1992). Invasins, the gene products of the *inv* gene loci in *Y. enterocolitica* and *Y. pseudotuberculosis* are optimally produced at 30°C rather than at 37°C (Isberg *et al.*, 1988; Young *et al.*, 1990). Clearly the regulation of virulence in yersiniae is more complicated than in shigellae with some virulence factors being optimally expressed at 37°C whilst others are shut-down.

Bordetella pertussis, *B. bronchiseptica* and *B. parapertussis* are closely related species, they all adhere to the ciliated cells in the upper respiratory tract of mammals and cause disease by expressing a number of virulence factors. *Bordetella pertussis* causes whooping cough in man while *B. parapertussis* causes a milder form of the disease, *B. bronchiseptica* is a pathogen of dogs and pigs (Aricò *et al.*, 1991). In the case of antigenic/phenotypic modulation the expression of many virulence genes of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* is regulated through the pleiotropic *bvg* (Bordetella virulence gene) locus, formerly known as *vir*. *Bvg* controls expression of subservient genes at the transcriptional level (Melton & Weiss, 1989). Genes under positive control by *bvg* are those encoding pertussis toxin (*B. pertussis* only), adenylate

cyclase toxin, haemolysin, dermonecrotic toxin, filamentous haemagglutin and fimbrae. The *bvgA* and *bvgS* genes encode a two-component regulatory system that responds to temperature (*bvg* activated genes are not expressed at low temperature, 22°C - 27°C) , and concentrations of magnesium sulphate and nicotinate (Lacey, 1960; Schneider & Parker, 1982; Armstrong & Parker, 1986) .

In the Gram-positive pathogen *Listeria monocytogenes*, expression of genes required for infection is controlled by temperature via the transcription regulatory protein Prf (Leimeister-Wachter *et al.*, 1992)

1.7.4 Relevence of temperature regulation to legionellae

Although legionellae are commonly found in man-made water systems (Bartlett *et al.*, 1983), natural surface water (Fliermans *et al.*, 1981), and even potable water (Colbourne & Dennis, 1989), the incidence of Legionnaires' disease is relatively low (accounting for only around 2% of community acquired pneumonias in the United Kingdom (Research Sub-Committee of the British Thoracic Association, 1987). Host factors, size of challenge dose, differences in the virulence between strains and presentation in a respirable aerosol are all factors in whether infection occurs. It is, however, conceivable that some aspect of the growth environment either selects for sub-populations of virulent legionellae, or stimulates the expression of a pathogenic phenotype, and when such an event occurs the potential for an outbreak exists. The notion of temperature as an environmental stimulus is of particular relevence to legionellae as these bacteria can experience a wide range of temperatures in the environment. Legionellae are thermotolerant organisms: *Legionella pneumophila* has been isolated from water between 5.7° and 63° C (Fliermans *et al.*, 1981); and has been shown to grow between 25° and 45° C (Wadowsky *et al.*, 1986). There is evidence for thermoregulation of gene expression in legionellae. Ott *et al.* (1991) reported that temperature influenced the expression of flagella in *L. pneumophila* and Edelstein *et al.* (1987) noted variation in the virulence of *L. pneumophila* which was

dependent on growth temperature.

1.7.5 Iron restriction as a regulatory stimulus

As mentioned earlier the availability of free iron *in vivo* is severely limited, hence iron restriction is a clear signal to a bacterium that it has invaded a vertebrate. Iron was recognised as a regulator of bacterial virulence in the 1930s, when Pappenheimer and Johnson (1936) demonstrated that it regulated diphtheria cytotoxin production. Likewise a number of bacterial toxins are produced optimally under iron-restricted conditions: toxin A of *Pseudomonas aeruginosa* (Bjorn *et al.*, 1978); diphtheria toxin (Pappenheimer & Johnson, 1936); shiga toxin (Dubos & Geiger, 1946); α-toxin of *Clostridium perfringens* (Pappenheimer & Shaskan, 1944); and tetanus toxin (Meuller & Miller, 1945). The virulence of *Neisseria meningitidis* is greatly increased by growth under iron-restriction at low pH (Brener *et al.*, 1981). There is evidence to suggest that iron-restriction has a role in selecting for mucoid strains of *Pseudomonas aeruginosa* in the lungs of individuals with cystic fibrosis (Ombaka *et al.*, 1983; Brown *et al.*, 1984).

Bacteria exhibit a global response to iron restriction, including the derepression of genes for siderophore synthesis and for the production of receptors necessary in the binding of the Fe^{3+}-siderophore complex and internalisation of the iron. Growth of *E. coli* under iron-limited conditions results in the production of under modified transfer RNA (tRNA) which has the net effect of increasing the expression of operons coding for the biosynthetic pathways of aromatic amino acids (Buck & Griffiths, 1982). Enterobactin, the siderophore produced by *E. coli*, is synthesised from chorismic acid by way of a branch of the aromatic biosynthetic pathway (Griffiths, 1987b). Outer membrane protein receptors involved in iron uptake are regulated by iron concentration in the environment. A number of pathogens including *Aeromonas, Escherichia, Klebsiella, Salmonella, Shigella and Vibrio* express such proteins, which are integral components of iron-uptake systems that involve siderophores, during iron-limited

growth. *Neisseria meningitidis* and *N. gonorrhoeae* are able to acquire iron from transferrin and lactoferrin. The iron-uptake systems of these bacteria do not appear to involve siderophores; however both these bacteria express new outer membrane proteins during iron restriction (Mietzner *et al.*, 1984; West & Sparling, 1985).

1.8 LEGIONELLAE IN THE ENVIRONMENT

Legionellae are most commonly, although not exclusively, associated with aquatic environments, and particularly where elevated temperatures prevail. *Legionella pneumophila* has been isolated from both man-made and natural water systems.

1.8.1 Legionellae and natural water systems

Legionella pneumophila has been isolated from natural surface waters: rivers and fresh water lakes (Fliermans *et al.*, 1981). Rivers and lakes within the blast zone of the Mount Saint Helens volcanic eruption were found to have higher concentrations of several species of *Legionella* than similar water courses outside the blast zone, in addition legionella numbers were consistently high in lakes receiving water from hydrothermal seeps (Tison *et al.*, 1983). Research personnel exposed to these environments reported "flu-like" symptoms and Pontiac fever was one of the diagnoses considered. The investigations led to the isolation and naming of a new species, *Legionella sainthelensis* (Campbell *et al.*, 1984).

An epidemiological survey in France resulted in the isolation of a new strain of *Legionella* from the water of a thermal spa. The microorganism was subsequently named as a new species, *Legionella gratiana* (Bornstein *et al.*, 1989).

1.8.2 Natural environments and legionella survival

Early ecological studies of *L. pneumophila* indicated that the microorganism was ubiquitous in the natural aquatic environment and was capable of surviving in a wide

range of environmental conditions (Fliermans *et al.*, 1981).

Legionella pneumophila has been isolated from habitats with temperatures ranging from 5.7°C to 63°C, pH values from 5.5 to 8.1, dissolved oxygen concentrations of between 0.3 and 9.6 mg l^{-1} and conductivities of 18 to 106 μS cm^{-1} (Fliermans *et al.*, 1981). An ecological study of the distribution of *Legionellaceae* in Puerto Rican waters has provided further data on the physicochemical characteristics of habitats from which legionellae could be isolated (Ortiz-Roque & Hazen, 1987). The sites sampled were marine, estuarine and fresh waters. These waters were polluted to varying degrees. Significant positive correlations were found between *L. pneumophila* densities and pH, concentration of phosphates or sulphates, or presence of other microorganisms. In contrast, negative correlations were found when salinity and ammonia concentration were compared with *L. pneumophila* densities.

1.8.3 Man-made water systems

The majority of legionellae are isolated from man-made water systems, particularly hot water systems in buildings and evaporative cooling applications (evaporative/wet cooling towers and evaporative condensers). This is, perhaps, not surprising since water temperatures commonly found in such systems (particularly in the days before increased awareness of the risk of Legionnaires' disease and the advent of guide-lines and legislation aimed at the prevention of legionella infections) are conducive to the growth of legionellae.

1.8.4 Man-made water systems and legionella survival

Legionella pneumophila multiplies in potable water between 25 to 42°C (Yee & Wadowsky, 1982; Wadowsky *et al.*, 1985) and suffers no loss of viability at 46°C with a decimal reduction times of 111 minutes at 50°C and 27 minutes at 54°C in pure cultures (Dennis *et al.*, 1984). Schulze-Röbbecke *et al.* (1987) obtained similar results in experiments where *L. pneumophila* was cultured in tap water with associated

microorganisms: recording decimal reduction times of 19, 6 and 2 minutes for 55, 57.5 and 60°C, respectively.

The first evidence implicating hot- and cold-water systems in large buildings as the source of legionellae, subsequently causing legionellosis, came from a hospital outbreak in Oxford (Tobin *et al.*, 1980). It was, however, later recognised that legionellae could be isolated from water systems where no disease had occurred (Dennis *et al.*, 1982; Wadowsky *et al.*, 1982). A PHLS survey of large buildings, such as hospitals and hotels, in England and Wales found that 56% of hot water systems and 13% of cold-water systems contained legionellae (Bartlett *et al.*, 1983; Public Health Laboratory Service, 1985). Moreover, a study carried out by Thames Water Authority indicated that *L. pneumophila* was present in 12% (determined by immunofluorescence) of treated water samples from the public supply. This study also found that *L. pneumophila* was present in 40% of treatment works serving outlets in London and from these results it was estimated that 60% of buildings in the capital could have contained legionellae at some time during the survey (Colbourne & Dennis, 1989).

The chemical and bacteriological characteristics of the water found to be positive for *L. pneumophila* in the survey was generally indistinguishable from those of the water in the whole supply area. However, two chemical parameters, the total organic carbon concentration and the concentration of zinc, were higher in legionellae-positive water samples (Colbourne & Trew, 1986). In a laboratory study where hot water samples were inoculated with *L. pneumophila* , supplementation of tap-water with 0.5 or 1 mg l^{-1} iron or zinc stimulated the growth of *L. pneumophila*, whereas higher concentrations, 10 or 100 mg l^{-1}, were inhibitory. Potassium enhanced legionellae growth at 1, 10 and 100 mg l^{-1}. In addition, of the 23 chemical parameters measured in the hot-water, only iron concentration correlated positively with *L. pneumophila* population size (States *et al.*, 1985).

1.8.5 Legionellae and evaporative cooling towers

Outbreaks of Legionnaires' disease have been associated with a variety of aerosol generators, including evaporative cooling towers, hot water systems, whirlpool spa baths, clinical humidifiers, supermarket vegetable sprays and fountains. However, in Britain almost all of the major outbreaks of Legionnaires' disease have been associated with evaporative cooling towers (see Table 1.1). Figures provided by CDSC (personal communication) indicated that over 60% of outbreak cases were associated with evaporative cooling towers.

Evaporative cooling towers were first implicated as sources of the causative microorganism of Legionnaires' disease and as vehicles for the aerosol transmission of the legionellae as the result of an Legionnaires' disease outbreak in Memphis, Tennessee in 1978 (Dondero *et al.*, 1980). Since then there have been numerous reports where evaporative cooling towers have been implicated as the source of outbreaks of Legionnaires' disease and of Pontiac fever. Cooling towers have also been implicated in non-outbreak (sporadic) cases of Legionnaires' disease (Bhopal *et al.*, 1991).

In a survey carried out by the PHLS in the early 1980s 50% of the cooling towers sampled were found to contain *L. pneumophila* (Public Health Laboratory Service, 1985). The majority of evaporative cooling towers associated with outbreaks of legionellosis are relatively small installations, having heat rejection capacities of less than 300 kW. The positive correlation between "small" cooling towers and legionellosis may be explained by the intermittent nature of the operation of small cooling towers relative to the continuous operation of large cooling towers. Moreover, small cooling towers have higher wet surface area to volume ratios which may result in increased biofouling (Bentham & Broadbent, 1993).

Table 1.1 Outbreaks of Legionnaires' disease associated with evaporative cooling towers in the United Kingdom.

Incident	Outcome	Reference
Construction site, 1981	6 cases; 1 death	Morton *et al.* (1986)
Dennistoun, Glasgow, 1984	33 cases	Ad-Hoc Committee (1986)
Hospital, Glasgow, 1985	16 cases	Timbury *et al.* (1986)
Hospital, Stafford, 1985	68 cases; 22 deaths	O'Mahony *et al.* (1990)
Industrial estate, Ramsgate, 1985	5 cases; 1 death	Communicable Disease Surveillance Centre (1985)
Police HQ, Lincoln, 1985	7 cases; 1 death	O'Mahony *et al.* (1989)
Town centre, Gloucester, 1986	15 cases; 1 death	Communicable Disease Surveillance Centre (1986)
Broadcasting House, London, 1988	70 cases; 3 deaths	House of Commons Employment Committee (1989)
Engineering plant, Bolton, 1988	33 cases	Mitchell *et al.* (1990)
Leicester Square, London, 1989	29 cases; 5 deaths	Communicable Disease Surveillance Centre (1989a)
South Kensington, London, 1989	5 cases; 1 death	Communicable Disease Surveillance Centre (1989b)
Army Camp, near Winchester, 1991	3 cases	Watson & Bezzant (1992)
Industrial site, Yorkshire, 1992	3 cases	Joseph *et al.* (1993)
Piccadilly Circus, 1993	3 cases, 1 death	Joseph *et al.* (1994)
Birmingham, 1994	8 cases; 1 death	Joseph *et al.* (1995)
Edinburgh, 1994	9 cases; 1 death	Communicable Disease Surveillance Centre (1994)

1.9 EVAPORATIVE COOLING

1.9.1 Principles of evaporative cooling

When water changes its state from liquid to vapour an input of energy is required. Evaporative or wet cooling towers are heat rejection devices which take advantage of this phenomenon to remove unwanted heat from a water circulation system.

In process cooling (*e.g.* air compressor, diesel engine, induction furnace, turbine), water is circulated from the cooling tower pond to the process equipment. A more common application of a cooling tower (certainly towers of the type associated with legionellosis) is in association with an air conditioning system where the cooling tower indirectly removes heat from the building.

During the summer cooling towers can only achieve temperatures approaching 25°C, however air conditioning systems require temperatures as low as 5°C. To attain the appropriate temperatures for cooling a refrigeration process is used. This absorbs low grade heat from the air conditioning system and transfers it as higher grade heat to the cooling tower circuit, at a temperature sufficient to take advantage of evaporative cooling. Water is circulated from the cooling tower to cool the refrigeration condenser of the air conditioning system allowing the refrigerant to be compressed and continue its cooling duty. The operating temperature of the majority of water cooled condensing units lies between 35°C and 45°C (Hill *et al.*, 1990). Thus, the water in the cooling tower circuit is at a temperature conducive for the growth of *Legionella* species.

1.9.2 Evaporative cooling tower design and operation

The principal features of the design and operation of evaporative cooling towers allow optimisation of heat transfer, by achieving maximum contact between air and water and minimisation of water use. Water is pumped from the pond to the process requiring cooling, where it picks up heat, it then moves on through the distribution system and

falls through the body of the tower. The water flow is then broken up by the tower packing, which maximises heat rejection, before returning to the pond having been cooled. Cooling towers recirculate water in order to reduce water consumption. Consequently, due to evaporation of water, there in an increase in the concentration of dissolved solids and other impurities. This build-up has to be controlled to allow efficient operation of the cooling tower. Regulation of dissolved solids involves purging and discharging this water to drain. Fresh make-up water is introduced to compensate, thus keeping the level of dissolved solids under control. The purge may be continuous, where a valve constantly bleeds water off the system, or controlled, on the basis of conductivity. The concentration of total dissolved solids (TDS) is proportional to the conductivity of the water, as monitored by a conductivity cell which activates the purge valve once a predetermined control level has been reached. A glossary of terms describing the main structural components and operation of evaporative cooling towers and a diagram demonstrating the components of a cooling tower are included in Appendix.

1.9.3 Contamination of cooling systems

Microorganisms may enter the cooling system water via the water supply, from the air, or during installation or alterations. The water falling through the tower entraps dirt and other particulate matter from the air stream, thus providing a moist, nutrient rich environment for microbial growth. Estimates suggest that a typical tower rejecting 700 kW and handling an airflow of 19000 l s^{-1} could receive around 100 kg of particulate matter from the air in 1000 hours of operation (Broadbent, 1993). Evaporative cooling towers are therefore capable of supporting high concentrations of legionellae and must therefore be treated with biocides to control microbial proliferation.

Cooling towers not only provide an excellent environment for the amplification legionellae but also their operation results in the aerosol formation of the water phase.

Any legionellae present in the recirculating water will subsequently be found in water droplets in the air around the cooling tower. Control strategies for evaporative cooling towers have been designed to minimise the colonisation of systems and to reduce quantity of water droplets (drift) exiting the tower. It should be noted that both of these control measures were initially introduced for quite different reasons. Biocides were originally used in cooling towers to minimise biofouling as this impairs heat transfer. At their advent drift eliminators were used to minimise the nuisance caused to people close to a tower who might be subject to a damp spray; drift loss of between 0.1 and 0.25% of the total water circulation was considered acceptable. Modern high efficiency drift eliminators are designed to reduce the numbers of small water droplets and cut water loss to between 0.005 and 0.001% of the recirculation (Hill *et al.*, 1990).

1.9.4 Alternatives to evaporative cooling

It has been estimated that there are around 100,000 evaporative cooling towers operating in the United Kingdom. They are highly efficient, such that, if they were to be replaced by non-evaporative coolers, such as finned tube air blast coolers the increased power demand would be around 800 MW. In addition, evaporative cooling can produce temperatures, commonly required by industry and in air conditioning systems, which are below ambient air dry bulb temperature. The only way of achieving these temperatures is by refrigeration but the cost can be over eight-fold greater than that of a cooling tower. Moreover, refrigeration requires higher energy consumption and uses chloro-fluro carbons (CFCs), a class of chemicals recognised as environmental pollutants. The efficiency and economic data indicate that evaporative cooling towers are essential and will continue to be installed and operated for the foreseeable future (Hill *et al.*, 1990). Only in buildings, such as hospitals, where there is potentially a large population susceptible to Legionnaires' disease is it appropriate to consider the use of alternative forms of cooling, such as air blast coolers or air cooled condensers.

The Joint Health and Safety Executive and Department of Health Working Group on Legionellosis (HSE/DoH WGL; 1992) in its First Report to the Health and Safety Commission and Chief Medical Officers concluded that wet cooling towers provide a valuable service and should present minimal risk when current guidance for installation and maintenance is adhered to. No recommendation was made to change from wet to dry cooling systems.

1.9.5 Legislation and regulation of cooling systems

The Health and Safety at Work etc. Act 1974 and the Control of Substances Hazardous to Health (COSHH) 1988 Regulations relate to the risk of legionellosis. The Health and Safety Executive (HSE), together with Local Authority Environmental Health Officers, are responsible for enforcing health and safety legislation relating to minimising the risk of Legionnaires' disease to both employees and the general public. In 1992 the Health and Safety Commission (HSC) introduced an Approved Code of Practice (ACoP) "The prevention or control of legionellosis (including Legionnaires' disease)" (HSE, 1991b) aimed at providing practical guidance with respect to the requirements of the relevant legislation. Moreover, on the 2nd of November 1992 legislation (The Notification of Cooling Towers and Evaporative Condensers Regulations 1992, SI 1992/2225) came in to force requiring operators of cooling towers and evaporative condensers to notify the relevant Local Authority of the existence of these cooling devices.

1.9.6 Advice and guidance on cooling system operation

Advice and guidance on maintaining systems to minimise the risk of legionellosis has been published by various bodies (*e.g.* Chartered Institution of Building Services Engineers, 1991; HSE, 1991a; NHS Estates, 1993). The guidance in these documents has been based on experience of systems put in place to control other problems such as impairment of heat transfer by biofouling. The guidelines offered, although logical and reasonable, are mainly based on empirical evidence and their efficacies have not been scientifically proven. In their First Report to the Health and Safety Commission and

Chief Medical Officers the Joint Health and Safety Executive and Department of Health Working Group on Legionellosis (1992) stated that: "Fundamental to the provision of comprehensive and sound guidance on control is a much improved understanding of the ecology of legionella in both natural and man-made environments." In order to enhance its ability to provide the best possible advice and guidance, the HSE has sought to obtain scientific data on the development and control of *Legionella pneumophila* in evaporative cooling towers. This information cannot be determined by field studies of operational cooling towers because it would be unethical and unsafe to allow large populations of infectious legionellae to develop in them. For this reason a project "Studies on the development and control of populations of infectious *Legionella pneumophila* in cooling towers" was set up at CAMR funded by the HSE (reference 1/HPD/126/145/91), to construct a microbiologically-contained cooling tower for subsequent use in the study of the growth of legionellae in evaporative cooling towers.

1.10 FACTORS INFLUENCING THE GROWTH OF LEGIONELLA IN COOLING TOWERS

Various studies have been carried out to examine the relationship between legionella growth and other environmental factors.

1.10.1 Temperature

In the most extensive survey of it kind carried out to date, Broadbent and colleagues (1993) studied the microbial ecology of 65 evaporative cooling towers in Australia over a three year period. They collected over 9000 samples and observed correlations between legionella densities and physicochemical, microbiological or operational characteristics of the systems. Temperature was found to be a major factor in legionella colonisation. No legionellae were isolated from ponds with temperatures of less than 16.5°C and logarithmic growth of legionellae was observed at 23°C and over. Tower usage was found to correlate positively with the number of legionellae in the

system. No correlation was found between pH, conductivity or total bacterial counts and legionellae concentrations (Bentham *et al.*, 1993; Broadbent, 1993).

1.10.2 Water chemistry and nutrients

In a similar, although less extensive, survey in Japan 82 samples were collected from 40 cooling towers and examined for legionellae. Positive correlations were observed between viable counts of legionellae and pond water temperature, pH or protozoan counts, but not with heterotrophic bacterial counts (Yamamoto *et al.*, 1992). A Finnish study of 30 cooling towers (Kusnetsov *et al.*, 1993) reported no association between water temperature or pH and the occurrence of legionellae in the systems. The presence of legionellae correlated negatively with total bacterial counts and that legionellae were more frequently isolated from waters of low nutrient content. No significant correlation was observed between legionella counts and the concentrations of phosphate, sulphate, chloride, calcium, chromium, copper, iron, magnesium, manganese, or zinc; nor was there any significant correlation with dissolved organic carbon, oxygen content, hardness or conductivity. Protozoa were not found in any of the systems (Kusnetsov *et al.*, 1993).

1.10.3 Evaporative cooling tower operation

Howland and Pope (1983) reported that the concentrations of presumptive *L. pneumophila* in water and sediments of cooling towers was dependent on cooling tower operation. The greatest density of presumptive *L. pneumophila* in the seasonally operated (April to October) cooling tower occurred in June for water samples and July for sediment samples; this was mirrored by an increase in basin water temperature and pH. Legionellae numbers decreased between August and November as did water temperature and pH. In the system which operated continually throughout the year water temperatures and pH values were found to be relatively constant as was the concentration of presumptive *L. pneumophila* in the sediment. However, the population of presumptive *L. pneumophila* in the planktonic phase was found to vary. Lowest

legionellae numbers occurred in the winter months, followed by an increase during spring with highest concentrations occurring in summer and autumn (Howland & Pope, 1983).

1.11 LEGIONELLA ASSOCIATIONS WITH OTHER MICROORGANISMS

Legionella species are not believed to able to grow in water without the presence of other microorganisms or extensive supplementation with nutrients, including iron salts, cysteine and α-ketoglutarate.

1.11.1 Algae

The first evidence that other microorganisms might support the growth of legionellae was reported by (Tison *et al.*, 1980), who demonstrated that *L. pneumophila* could grow in minimal salts medium in association with a cyanobacterium (*Fischerella* sp.) at 45°C over a pH range of 6.9 to 7.6. The authors concluded that the growth of *L. pneumophila* in these co-culture experiments was dependent on the extracellular release of substrates, by *Fischerella* sp., when the alga was actively photosynthesising. In addition green algae, including a *Chlorella* sp., *Gleocytis* sp. and *Scenedesmus* sp., have been shown to support the growth of *L. pneumophila* in basal salts medium (Hume & Hann, 1984).

1.11.2 Protozoa

Rowbotham (1980) first reported the growth of *L. pneumophila* in amoebal trophozoites and their incorporation into amoebal cysts. Subsequently, substantial amounts of research have been carried out on legionella-protozoa associations. A number of studies have confirmed Rowbotham's original observations (Tyndal & Domingue, 1982; Anand *et al.*, 1983; Fields *et al.*, 1984; Newsome *et al.*, 1985; Rowbotham, 1986; Wadowsky *et al.*, 1988). Five genera of amoebae (*Acanthamoeba, Naegleria, Hartmannella, Vahlkampfia,* and *Echinamoeba*) and one genus of ciliated protozoa (*Tetrahymena*) have

been shown to be able to support the intracellular growth of *L. pneumophila* (Fields, 1993). This association between legionellae and amoebae has important practical implications. Since intracellular growth of legionellae within amoebae has been reported to protect legionellae against the bactericidal effects of chlorine (King *et al.*, 1988; Kuchta *et al.*, 1993). Kilvington and Price (1990) demonstrated that *L. pneumophila* could survive exposure to 50 mg l^{-1} of chlorine within the cysts of *Acanthamoeba polyphaga*. In addition, Barker *et al.* (1992) suggested that growth within *A. polyphaga* might modify the susceptibility of *L. pneumophila* to commonly used biocides.

1.11.3 Bacteria

A number of associations, both synergistic and antagonistic, have been reported between *Legionella* species and other bacteria. The growth of *L. pneumophila* was shown to be supported by the growth of environmental microorganisms (Stout *et al.*, 1985). Wadowsky and Yee (1983) observed satellite growth of *L. pneumophila* around colonies of *Flavobacterium breve* on cysteine deficient medium and from this observation concluded that *F. breve* could be providing *L. pneumophila* with cysteine or some related compound. Two years later these same authors reported a similar growth supporting activity by three non-legionellae pigmented water isolates (Wadowsky & Yee, 1985).

Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Bacillus sp. and viridans group *Streptococci* (normal respiratory bacterial species) were shown to inhibit the growth of *L. pneumophila* (Carrington, 1979). Similarly, Flesher *et al.* (1980) reported the inhibition of *L. pneumophila* growth by human pharyngeal flora. *Legionella dumoffii* has been shown to be inhibited by *Aeromonas hydrophila, A. salmonicida, Flavobacterium meningoscepticum* and *Pseudomonas aeruginosa* (Paszko-Kolva *et al.*, 1991, 1993). Toze *et al.* (1993) have demonstrated inhibition of *L. pneumophila* by *Aeromonas* sp. Members of *Enterobacteriaceae,*

Pseudomonadaceae and *Vibrionaceae* have been shown to suppress the growth of legionellae (Gomez-Lus *et al.*, 1993). This study showed that bacteriocins produced by *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Aeromonas* spp. could suppress the growth of legionellae. *Legionella* species can be inhibited by a variety of heterotrophic plate count (HPC) bacteria isolated from chlorinated drinking water (Toze *et al.*, 1990). Of the hundred strains tested 16 inhibited the growth of *L. pneumophila.* The inhibitory strains identified were *Pseudomonas vesicularis, P. paucimobilis, P. maltophilia, Vibrio fluvialis* and members of the genera *Aeromonas* and *Pseudomonas.*

1.12 BIOFILMS

Growth in biofilms constitutes an important facet in the bacterial life cycle. It provides nutritional advantages and may help to protect microorganisms against adverse environmental conditions (Fletcher, 1979). Indeed, sessile enteric bacteria are more metabolically active than their planktonic counterparts (Hendriks, 1974). Costerton *et al.* (1981) proposed that once bacteria are attached to surfaces they produce copious amounts of extra-cellular polymer matrices often composed of fibrillar polysaccharide with the possible inclusion of some globular glycoproteins (glycocalyx; Sutherland, 1977). The organisation of bacteria into biofilms makes them more recalcitrant to inactivation by biocide (LeChevallier *et al.*, 1988; Keevil *et al.*, 1990) and has obvious implications for control of legionellae. Protection from biocidal activity may occur for a number of reasons: the glycocalyx may exclude or interfere with the action of the biocide; the glycocalyx or outer layers of the biofilm may adsorb the active agent; the physicochemical environment that the bacteria are exposed to in a biofilm may influence gene expression and/or the phenotype expressed by the bacteria, and this in turn may lead to enhanced resistance to chemical inactivation (Brown & Gilbert, 1993). Biofilms have been shown to harbour a number of pathogenic bacteria, including *L. pneumophila* in water system (Keevil *et al.*, 1989, 1995)

Although legionellae are known to survive and multiply in amoebae and other protozoa (see Section 1.11.2), *L. pneumophila* has been shown to grow extracellularly as part of a mixed biofilm (Rogers & Keevil, 1992). The dissolved oxygen concentration in biofilms can be considerably lower than in the bulk water phase (Costerton *et al.*, 1994); this may have important consequences for the survival of legionellae in aerated water systems such as cooling tower ponds.

Legionellae have been isolated in high numbers from sediments and slimes in cooling water systems (Colbourne & Dennis, 1988). A number of laboratory studies have demonstrated that legionellae are able to grow in biofilms on a variety of plumbing material surfaces including aluminium, copper, galvanised steel, stainless steel, glass, wood, polyvinyl chloride, and rubber (Schofield & Locci, 1985); polyvinyl chloride, brass and copper (Bezanson *et al.*, 1992); copper, glass, polybutylene and natural rubber (West, *et al.*, 1989, 1990); copper, polybutylene and polyvinyl chloride (Rogers *et al.*, 1994). The level of colonisation is dependent on the plumbing material. Natural rubber is heavily colonised while copper is inhibitory to growth of legionellae (Schofield & Locci, 1985; West, *et al.*, 1989, 1990). These reports have clear implications for the survival and growth of legionellae in man-made water systems and for the resistance of these bacteria to biocides and other methods of control and disinfection. Furthermore, Wright *et al.* (1991) demonstrated that sessile *L. pneumophila* were more resistant to in activation by biocide than their planktonic counterparts.

1.13 CONTINUOUS CULTURE

1.13.1 Bacterial growth

As a prelude to a description of the theory of continuous culture it is necessary to discuss some basic aspects of bacterial growth which are pertinent to the topic.

The following simple equation can be used to represent exponential growth of bacteria

$$\frac{1}{x}\frac{dx}{dt} = \frac{d(\log_e x)}{dt} = \mu = \frac{\log_e 2}{t_d}$$

where x is the concentration of bacteria (dry weight of bacteria/unit volume) at time t, μ is the specific growth rate (*i.e.* the rate of increase/unit of bacteria concentration) and t_d is the doubling time.

Monod (1942) first showed that there is a simple relationship between the specific growth rate (μ) and the concentration of an essential growth substrate. Growth rate is proportional to the concentration of an essential substrate when this is low but reaches a limiting saturation value at high substrate concentrations according to the equation

$$\mu = \mu_{max}[s/(K_s+s)],$$

where s is the substrate concentration, μ_{max} is the maximum growth rate, K_s is the saturation constant numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\mu_{max}$. It follows from this that the exponential growth rate can occur at any value between zero and μ_{max}. This fact is of crucial significance to the theory of continuous culture (Monod, 1950; Novick & Szilard, 1950).

Monod (1942) also showed that there is a simple relationship between growth and utilisation of substrate. This occurs in its simplest form when the growth medium contains a single carbon source; under such conditions the growth rate is a constant fraction, Y, of the rate of substrate utilisation:

$$dx/dt = -Yds/dt,$$

where Y is known as the yield constant. Therefore over any finite period of growth

$$Y = \frac{\text{weight of bacteria formed}}{\text{weight of substrate used.}}$$

1.13.2 Culture systems

The techniques for culturing bacteria, or indeed any microorganisms which can be grown in submerged conditions, can be said to fall into two broad categories: closed culture systems and open culture systems. Closed culture systems are perhaps the most commonly used in microbiology and are commonly referred to as batch cultures. A closed system has no input or output of materials other than gases. Closed culture systems formed (and still form) the main stay of cell culture for over a century until the advent of continuous culture techniques in 1950 offered a viable alternative. The major drawback of batch culture techniques is that the growth environment is in continuous flux, which is an inevitable consequence of microbial growth. Substrates in the growth medium are metabolised and waste products of microbial growth, including secondary metabolites which accumulate in the medium, can lead to alterations in the pH and redox potential of the growth environment. In addition, the nature of the bacterial population itself varies with time, starting with the lag phase in which the bacteria adapt to the new environment (it should be noted that the method of preparation of the inoculum influences both the extent and duration of this initial phase), this is followed by a time of exponential growth, cessation of growth occurs when either the essential nutrients have been exhausted or there is an accumulation of toxic metabolites or there is a change in the ionic equilibrium, especially pH: this is termed stationary phase. The final phase in the growth of a batch culture is the decline phase (a period of negative growth) in which the rate of cell death exceeds the growth rate.

An open culture system is defined as one in which there is both an input of substrate (growth medium) and an outflow of spent medium, biomass and microbial products. The two distinct manifestations of open culture systems are plug(piston)-flow and

completely-mixed culture systems. The former simulates batch culture in an open system and offers no additional control over the growth environment, its main feature is that the phases of batch culture which are temporally separated are also spatially separated in plug-flow reactors. The chemostat is the simplest form of the completely mixed reactor. The chemostat was described separately by Monod (1950), and Novick and Szilard (1950), the name Chemostat being coined by the latter authors; Monod named his apparatus as a Bactogen. Chemostat is the term which has been adopted into general use. The existence of an alternative system warrants mention at this point. Novick (1955) described the two types of continuous culture systems based on the completely mixed vessel principle, these differed on how control of growth rate is achieved.

Firstly, the chemostat in which control of the growth rate is achieved by a fixed flow of medium into the culture vessel at a value lower than the maximum growth rate of the culture. The other variant is designed to maintain a constant biomass, which it does by employing a monitoring system such as a light source and photocell to measure a change in the density of the culture (Bryson & Szibalski, 1952). The monitoring system is used to control the rate at which medium is fed into the culture vessel. Such systems are termed turbidostats and as the name suggests are able to maintain a constant culture density at a pre-determined value.

1.13.3 The theory of chemostat culture

The principle features of the chemostat are a culture vessel in which the microorganisms are grown, sterile growth medium is fed into this vessel at a steady flow rate (f) and culture emerges from it at the same rate, a constant-level or weir keeps the culture volume (v) constant (Herbert *et al.*, 1956). The contents of the culture vessel have to be efficiently stirred in an attempt to achieve the ideal of complete mixing, so that the growth medium is instantaneously and uniformly dispersed throughout the vessel. Assuming ideal mixing the residence time of cells in the vessel

will be determined by the ratio of the flow-rate to the culture volume, this is known as the dilution rate and is defined as $D = f/v$, its units being reciprocal hours (h^{-1}). The bacteria in the culture vessel are growing at $\mu = \log_e 2/t_d$ and simultaneously being washed out at a rate determined by $Dx = -Dx/dt$, the wash-out rate, where x is the concentration of bacteria in the vessel the net rate of increase of the concentration of bacteria is given by:

$$\text{increase} = \text{growth} - \text{wash-out}$$

$$dx/dt = \mu x - Dx.$$

Therefore if $\mu > D$, dx/dt is positive and the concentration of bacteria will increase, while if $D > \mu$, dx/dt is negative and the culture will be washed out of the vessel. When $\mu = D$, dx/dt and x are constant, *i.e.* a steady-state in which the concentration of bacteria remains constant will be attained. The chemostat is a self-stabilising system. Herbert *et al.* (1956) reported that E.O. Powell had determined that starting from any initial values of biomass or substrate concentration that a system will adjust itself to steady state as this is the only stable state for the system. They illustrated with the example that given a system which has been freshly inoculated, when x is very small, s is near to the concentration in the supply vessel (s_r) and $\mu > D$. The concentration of bacteria will increase, but due to a reduction in the substrate concentration the specific growth rate will decrease until μ becomes equal to D. At this point the combined rates of substrate loss and consumption just balance the rate of substrate addition and the system exhibits no tendency to change. The system is self-adjusting in that any small accidental fluctuations from the steady-state will set up opposing reactions which will restore the status quo.

Chemostat theory as elucidated by Monod (1950) and Novick and Szilard (1950) predicts that any number of steady states can be obtained at dilution rates between zero and the critical dilution rate. This was proved experimentally by Herbert *et al.* (1956).

1.13.4 Equations describing chemostat culture

Herbert *et al.* (1956) derived a series of equations to completely describe continuous culture, defining the upper limit of the dilution rate, D_c and the doubling time, t_d. The first four of these equations, although derived differently, are similar to those derived by Monod (1950).

$$dx/dt = x\{\mu_{max}(s/[K_s + s]) - D\}$$

and

$$ds/dt = D(s_r - s) - (\mu_{max}x)/Y(s/[K_s + s]).$$

They went on to describe equations which pertain to the steady-state, in which the equilibrium values of x and s, $\bar{x}$ and $\bar{s}$ respectively

$$\bar{s} = K_s(D/[\mu_{max}-D])$$

$$\bar{x} = Y(s_r - \bar{s}) = Y\{s_r - K_s(D/\mu_{max} - D)\}.$$

In the steady state the specific growth rate equals the dilution rate

$$\mu = \log_e 2/t_d = \mu_{max}(\bar{s}/[K_s + \bar{s}]) = D$$

therefore the doubling time, $t_d = \log_e 2/D = 0.693/D$.

The critical value of the dilution rate, D_c, above which complete wash-out occurs is given by

$$D_c = \mu_{max}(s_r/[K_s + s_r]).$$

All dilution rates greater than D_c will result in all the bacteria being completely washed out of the reaction vessel and obviously no steady-state culture is possible under such conditions.

1.13.5 Experimental uses of chemostat culture

The chemostat permits biomass growth rate to be controlled with no change in the environment other that the concentration of the growth-limiting nutrient. This is a distinct advantage over batch culture where growth rate can only be altered by qualitative changes in nutrition or quantitative changes in the physicochemical environment such as temperature or pH value. These methods can introduce other effects which may mask growth rate effects, *e.g.* change in temperature can independently affect the growth rate and the RNA content of bacteria (Hunter & Rose, 1972). Secondly, chemostats are able to maintain substrate limited growth at a constant growth rate, a condition which can only be attained transiently in batch culture and only in concert with a changing growth rate. This allows investigation of an increased variety of bacterial growth states. The third purpose of chemostat culture, and the most pertinent to the experimental programme described in this thesis, is the ability of chemostats to fix the growth rate while the growth environment is altered. This permits the experimenter to distinguish the effects of growth rate change from those of environmental change (Pirt, 1975).

In summary, utilising continuous culture allows the maintenance of steady state growth at almost any growth rate (below a critical value) for as long as is required. In addition to providing a constant chemical environment, chemostat culture allows the growth rate to be fixed whilst environmental parameters are changed (Monod, 1950; Novick & Szilard, 1950; Herbert *et al.*, 1956). For these reasons the chemostat was chosen as the most appropriate tool for investigating the influence of the growth environment on the physiology and virulence of *L. pneumophila.*

1.14 INTRODUCTION TO EXPERIMENTAL WORK

This thesis essentially describes an investigation of the role of environmental factors in influencing the growth, physiology and virulence of the bacterial pathogen *L. pneumophila*. The course of this study followed two different routes both using distinct culture systems to provide new information on the behaviour of this pathogen. The first series of experiments to be described involve the use of chemostat culture to investigate the role of two important environmental parameters, temperature and iron-restriction, in the regulation of the physiology and virulence of *L. pneumophila*. Changes in culture temperature and the nature of the limiting nutrient inevitably result in changes in growth rate if bacteria are grown in batch culture. The use of continuous culture, however, allowed the growth rate to be held constant throughout the experiments thus allowing the effect of the variables under investigation to be viewed in isolation. Control systems in the chemostat maintained other conditions constant.

The aim of the second phase of the study was to investigate which environmental factors effect the growth of *L. pneumophila* in evaporative cooling towers. Since there are reasons to believe that fully virulent *L. pneumophila* do not necessarily behave as non-virulent strains, *e.g.* strains associated with human disease survive better in air (Dennis & Lee, 1988), it was considered essential to study the growth of a strain of *L. pneumophila* which was known to be pathogenic for man. However, it is too hazardous to allow normal cooling towers be to colonised with pathogens and it would be unethical to inoculate such systems with infectious legionellae. It was, therefore, decided that the study would be carried out using a microbiologically contained cooling tower, in which large populations of infectious *L. pneumophila* could develop without causing any hazard. This cooling tower, which will be described in more detail later, was designed to accurately model the construction and operation of operational systems in every respect, but to do so under microbiological containment. A comprehensive

data acquisition and logging system was designed and installed to continuously monitor the growth environment in the cooling tower.

The cooling tower experiments represent a quite different approach from the chemostat. In the latter specific factors which are known to influence the physiology and virulence of other bacteria were investigated and this was done in a reductionist manner with the factors studied in isolation using pure cultures of *L. pneumophila*. In contrast the cooling tower investigation aimed to create a simulated real-world situation and to investigate correlations between the growth of legionellae in the system and the growth environment. The only external control exercised on the cooling tower came from running it in a variety of operational scenarios, other than that the experimental approach was to closely monitor the physical, chemical and biological environment in the system. However, similarities exist between the two culture systems. The cooling tower, in common with commercial cooling towers, was open to contamination from both the make-up water and the air stream (see Appendix), thus it represents a type of fed batch culture system, and in this respect resembles a kind of pseudo-chemostat where not only nutrient but other microbial species are continuously added to the system. Continuing this analogy, the chemical environment in the system is controlled to some extent by controlling the concentration of dissolved solids by purging water from the tower based on the conductivity of the water. This type of dynamic system with constant addition (from the make-up water and air) and loss (from the purge) of biotic and abiotic matter from the ecosystem and a fluctuating physicochemical environment more closely mimics growth conditions experienced by legionellae in their natural environment than in conventional laboratory culture. This experimental system facilitates a more comprehensive investigation of how *L. pneumophila* behaves in one of its most significant environmental niches.

In addition to the work described above, a section of this thesis deals with the development of a rapid phenotypic identification system for *Legionella* species based on

substrate utilisation. This system was also demonstrated to provide important metabolic information about the genus *Legionella*.

CHAPTER 2: MATERIALS AND METHODS

2.1 CONTINUOUS CULTURE

2.1.1 Strains used in continuous culture experiments

Two strains of *Legionella pneumophila* serogroup 1 monoclonal subgroup Pontiac were used in this study. The first strain, 74/81 was isolated during a field survey carried out by Dr. R.B. Fitzgeorge and Dr. P.J.L. Dennis of the Public Health Laboratory Service Centre for Applied Microbiology and Research (PHLS-CAMR) in the early 1980's from a source not associated with an outbreak; the second strain, Corby was an isolate associated with an outbreak of Legionnaires' disease (originally supplied to the laboratory by Dr. R.A. Swann of the John Radcliffe Hospital, Oxford). Both strains were stored as individual aliquots at -80°C, and were grown on Buffered Charcoal Yeast Extract (BCYE) agar (Edelstein, 1981) for 72 hours at 37°C prior to inoculation of the chemostat.

2.1.2 Continuous culture medium

The medium used was ACES-buffered chemically defined medium (ABCD; Pine *et al.*, 1986).

2.1.3 Continuous culture apparatus: chemostat, control/monitoring system and containment

Due to the risk of aerosol dissemination of *L. pneumophila* in the event of a failure in the integrity of the vessel, the chemostat (Plate 2.1) was housed in a specially constructed Class III microbiological safety cabinet (Plate 2.2). This practice resulted in operating difficulties, adding a further level of complexity to the supply and effluent lines, and requiring all electrical connections to be made through a pressure resistant interface. Nevertheless, this level of containment was viewed as being absolutely

Plate 2.1 Chemostat.

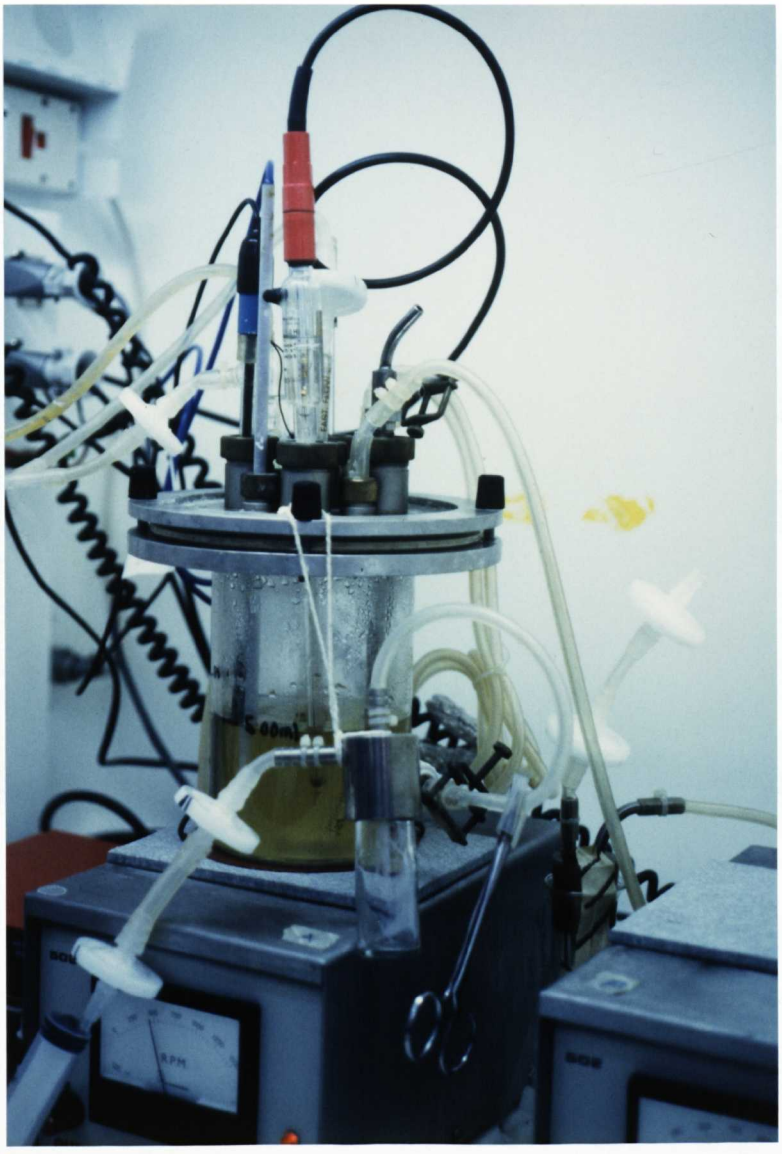
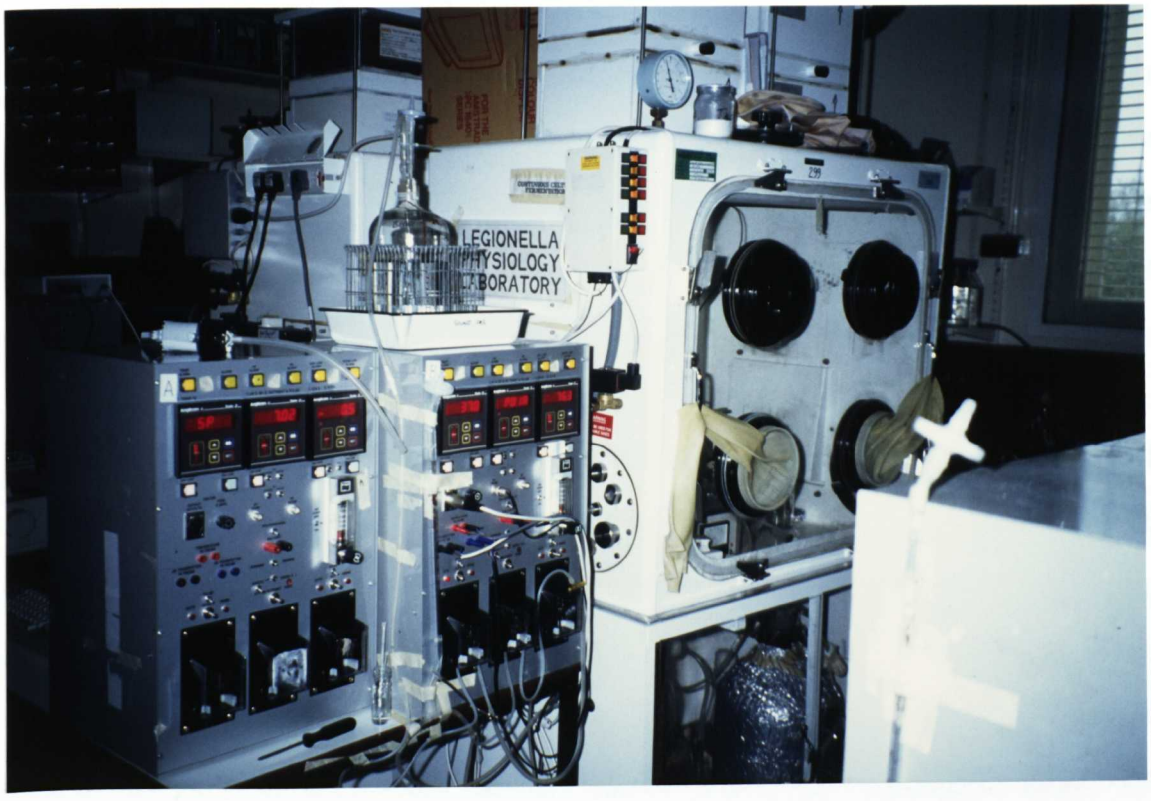


Plate 2.2 Class III safety cabinet and Anglicon Controllers.



necessary due to the high cell density of this pathogen in the chemostat.

A modified one litre fermenter (LH Fermentation, Stoke Poges) was used as the chemostat vessel. The modifications were the addition of two side arms (to act as entries for the weir and the sample line) and a specially constructed titanium top plate, which replaced the original made of stainless steel, so as to facilitate an investigation of iron-limited growth in *L. pneumophila*. This modification assured that no ferrous metal was in contact with either the culture or the medium. The working volume of the chemostat was maintained at 500 ml by use of a weir, from which culture was pumped out to the effluent bottle.

Probes used to monitor the physicochemical properties of the culture entered through the top plate, these were: a glass sheathed platinum resistance thermometer (Rt) (Brighton Systems, Newhaven); an Ingold gel filled pH electrode (Mettler-Toledo Ltd., Leicester); and a galvanic oxygen electrode (Uniprobe, Cardiff). The temperature probe also provided a compensation signal to the pH probe. All the probes were connected via an interface panel on the cabinet wall to Anglicon Microlab Fermentation Controllers (Brighton Systems, Newhaven) which regulated the temperature, pH and dissolved oxygen tension (DOT) by feed back control. The culture was heated by a heater pad below the chemostat. To achieve a constant temperature of 24°C it was necessary to integrate a "cold finger" into the top plate. Cold water was circulated through this from a W6 cooler unit (Grant, Cambridge), and the heater pad under the control of the Anglicon controller maintained the culture at 24°C.

Control of pH was effected by activation of a dosing pump which added sterile 1 M potassium hydroxide when the pH dropped below the set point (6.9), with the broth acting as acid titrant (the pH of ABCD broth is 6.5); this avoided the oscillation of pH that competing acid and base feeds can cause when such control is used.

Sterile medium was supplied to the chemostat by a peristaltic pump (Watson-Marlow, Falmouth) through an anti-growback device. The rate of medium addition, which determined the dilution rate (D), was set by varying the speed of rotation of the medium pump and was monitored daily by timing the delivery of a known volume from a flow meter integrated into the medium supply line. Pine *et al.* (1986) reported that the cysteine concentration ABCD broth rapidly decreased due to oxidation, and that the rate of depletion was increased in the presence of light. To prevent this and to reduce the potential for the production of toxic oxygen species the medium was held in an aspirator coated in aluminium foil, to exclude light, and oxygen-free nitrogen was bubbled through the medium prior to connection to the chemostat. Spent culture was pumped out with a similar pump to a receiving aspirator.

All flexible tubing used in the system was medical grade silicone (Sterilin Ltd., Hounslow, Middlesex), and was rinsed with deionised water prior to sterilisation.

Air filtered through an Acro 37TF 0.2 μm air filter (Gelman Sciences, Ann Arbor, Michigan, USA) was introduced over the surface of the culture via an entry port on the anti-grow back device. This practice was adopted to help prevent grow back in the medium line. For rapid gassing of the medium with nitrogen or air during calibration of the oxygen electrode, and deoxygenation of the medium a sparger was incorporated. Air exiting the chemostat passed through a condenser to remove moisture before venting through a similar filter. Aeration was accomplished by vortex mixing, which proved adequate and avoided the frequently encountered problem of foaming; consequently, there was no need to add antifoaming agents which may have affected the physiology or the cell walls of the bacteria. Vortex mixing was sufficient due to the low respiration rate of *L. pneumophila*.

2.1.4 Operation of chemostat and culture conditions

The chemostat was assembled, with all the probes and lines in place and then the

integrity of the vessel was tested both before and after autoclaving by applying a positive pressure using hand-operated bellows and looking for leaks causing bubbling at joints sprayed with detergent solution.

The pH probe was standardised against pH 4 and 7 buffers (Russell, Auchtermuchty, Fife) prior to sterilisation; the oxygen electrode was calibrated *in situ*, after the vessel was autoclaved (autoclaving accomplished the necessary deoxygenation of the electrolyte in the electrode) by bubbling oxygen-free nitrogen (British Oxygen Company) into the broth to set the zero and then bubbling air into the broth, to give 100% of air saturation. The medium in the vessel was replaced before inoculation, since the aeration of the broth would have lead to the formation of toxic oxygen species.

Previous chemostat experiments using several different levels of DOT indicated that maximal biomass concentration occurred at a DOT of 4% of air saturation at 30°C [equivalent to 0.31 (mg O_2) l^{-1}] (Araujo, Dennis & Keevil, unpublished results). This is consistent with reports of oxygen toxicity in *L. pneumophila* (Locksley *et al.*, 1982; Pine *et al.*, 1986). This oxygen concentration was used throughout the experiments. Since the DOT is influenced by temperature the oxygen concentration was maintained at 0.31 mg l^{-1} during growth at 24°C and 37°C by adjusting the DOT to 3.6% and 4.5% of air saturation, respectively. To promote growth the medium was deoxygenated with nitrogen until the DOT decreased to 4% of air saturation, prior to inoculation. The DOT was kept low by restricting agitator speed until growth was established. An oxygen concentration of 0.31 mgl^{-1} (equivalent to 4% of air saturation at 30°C) was maintained through feedback control of the agitation rate. After inoculation the culture was grown in batch until the turbidity (OD_{540}) reached 0.4. Continuous culture was initiated at a dilution rate (D) of 0.03 h^{-1}, and then increased to 0.08 h^{-1} (equivalent to a mean generation time, MGT, of 8.7 h) when the turbidity had increased to approximately 1.0. A dilution rate of 0.08 h^{-1} was used throughout experiments.

Turbidity (OD_{540}) and cell dry weight were used to assess when steady state had been achieved.

2.1.5 Culture sampling

Small samples (20 ml maximum) were withdrawn from the chemostat through the sample port into a sterile universal bottle. Large volumes of steady-state cultures were collected by disconnecting the effluent line from the receiving bottle and connecting it to a sterile collection vessel which was surrounded by ice.

Three independent chemostat experiments were carried out with strain 74/81 as the inoculum and two with strain Corby. A fresh inoculum was used for each experiment.

2.1.6 Culture monitoring

Culture purity was checked daily by plating on to BCYE agar and BCYE minus L-cysteine agar, *L. pneumophila* being auxotrophic for cysteine. Gram stains of culture were carried out routinely. Unstained smears were also viewed by differential interference contrast (DIC) microscopy (both transmission and episcopic illumination were used). At intervals the culture was also examined by an indirect immunofluorescence assay (IFA) using rabbit anti-*L. pneumophila* serogroup 1 antiserum and fluorescene isothiocyanate (FITC)-linked anti-rabbit immunoglobulin (Ig; reagents obtained from the PHLS Division of Microbiological Reagents and Quality Control, Colindale, London). The procedure followed for the IFA was: a small sample of culture was centrifuged and the resulting pellet resuspended in 2% (v/v) formalin in phosphate buffered saline (PBS) to give a suspension of only just visible turbidity. An aliquot (5 μl) of this suspension was placed in a well on a PTFE-coated multispot microscope slide, and dried in air at 37°C for 20 minutes. It was fixed in acetone at room temperature for 15 minutes. Antibody (5 μl) was added, and this was incubated at 37°C for 1 hour in a moist atmosphere. The slide was washed twice with PBS and once with distilled water (5 minutes for each wash), blotted and then dried at

37°C for 10 minutes. Anti-rabbit Ig FITC conjugate (5 μl) was added and incubated at 37°C for 30 minutes in a moist atmosphere (in the dark). The slide was washed in PBS and distilled water as before and then dried. The slide was then mounted in fluorescence preserving glycerol/PBS mounting medium (Citifluor Limited, London), and viewed by epi-illumination using ultraviolet light of 336 nm wavelength.

The optical density of culture samples was measured at 540 nm with a SP 500 spectrophotometer (Pye Unicam, Cambridge), samples were diluted with physiological saline when the OD_{540} was higher than 1.0. Biomass was assessed by filtering 10 ml of killed culture (1% [v/v] formaldehyde solution for 1 hour) through a pre-dried, pre-weighed 0.2 μm nylon membrane filter (Gelman Sciences, Ann Arbor, Michigan). The filtered samples were washed with 10 ml distilled water and then dried in a microwave oven until a constant weight was achieved. Estimations were done in triplicate and dry weights were calculated by differential weighing.

Total viable counts were determined by plating on to BCYE agar in duplicate and incubating at 37°C. The numbers of bacteria in the culture were determined using a Helber counting chamber on a phase contrast microscope. At least 200 bacteria were counted per slide and determinations were done in triplicate.

2.1.7 Chemical analysis of culture filtrates and medium samples

Analysis of amino acids (with the exceptions of tryptophan and cysteine) was performed using a 4400 dedicated amino acid analyser (LKB, Cambridge). This comprised of a lithium cation exchange separation system with post column reaction with ninhydrin and detection at 440 nm and 570 nm on a recorder and spectra physics integrator (Phillips, Cambridge). Buffers and reagents were obtained from Pharmacia (Milton Keynes) or Sigma (Poole). Calibration was carried out before each run using mixed physiological fluids standards (Sigma, Poole). Separation and detection of tryptophan was carried out by reverse-phase HPLC using a phosphate/citrate mobile phase (0.2 M Na_2HPO_4; 0.1

M citrate; methanol at 3:5:2) at pH 4 on a Hypersil ODS RP column. Detection was effected at 280 nm using a variable wavelength detector and recorder (Phillips, Cambridge). Reagents were obtained from Sigma (Poole) and BDH (Eastleigh). Samples were compared with freshly prepared tryptophan standard solutions. Cysteine concentration was determined colorimetrically using modification of the method of Gaitonde (1967).

The millimolar concentrations of metals were determined by flame atomic absorption spectrometry on a PU-9100 spectrophotometer (Philips, Cambridge); flame emission was used to detect potassium. Micromolar concentrations of iron were detected with an electrothermal ioniser attached to the PU-9100 spectrophotometer. The facility of the electrothermal ioniser only became available later in the study at a time which fortuitously coincided with the investigation of iron-limited growth.

Phosphate concentrations were determined by the Molybdenum Blue method (Vogel, 1983). Ammonium ion concentration was measured using a continuous flow Auto Analyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants.) as described by Gordon *et al.* (1978).

2.1.8 Electron microscopy

Cells obtained from the chemostat were left in 0.2% (v/v) formaldehyde for 2 hours, applied to formvar/carbon-filmed, 400 mesh copper EM specimen grids, and negatively stained with 1% (w/v) sodium silicotungstate. Alternatively, cells were fixed in 2.5% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide for embedding, thin sectioning and staining by the silver proteinate method of Thiéry (1967). Specimens were examined in a Philips EM 400T transmission electron microscope operated at 80 kV.

2.2 GROWTH LIMITING NUTRIENT

2.2.1 Modified continuous culture medium

The medium used in this study was a modification of ABCD broth in which the concentration of all the amino acids with the exceptions of serine and cysteine were reduced by 50%. This new formulation is referred to hereafter as MOD1.

2.2.2 Amino acid supplementation

Sterile solutions of each of these amino acids were added to the culture vessel sufficient to return the concentration of the amino acid under test to that in ABCD broth, and this concentration was maintained for at least 24 hours. An adequate time period was allowed between each amino acid addition to allow the concentration of the previous one to have decreased to a negligible level due to wash-out.

Efforts were made to use the most pure sources of the amino acids under investigation; to that end chromatographically homologous (CHR) preparations of arginine, proline, phenylalanine, threonine and tyrosine were obtained from BDH (Eastleigh, Hants.). The reported purity of these preparations was that the maximum contamination with foreign amino acids was 0.3% (w/w). Asparagine was not available in chromatographically pure form, but the preparation obtained from BDH had a maximum of 0.3% (w/w) foreign amino acids (the same purity as the other amino acids). With the exception of tyrosine the amino acid supplement pulse were dissolved in distilled water and sterilised by passing through a 0.22 μm filter (Sartorius, Gottingen, Germany). Since tyrosine is only sparingly soluble in water it was dissolved in KOH (0.14 M) and sterilised using a Millex-FG 0.22 μm acid/base resistant filter (Millipore, Molsheim, France).

The biomass concentration was monitored by optical density and dry weight measurements as described previously (2.1.6).

The results obtained indicated that tyrosine was the limiting nutrient so to confirm this the medium was changed from MOD1 to ABCD broth, steady-state allowed to re-establish and further supplementations carried out. A volume of tyrosine solution was added to the medium reservoir sufficient to double the concentration of tyrosine in the ABCD broth, to 0.56 mM. When a new steady-state was achieved a supplement of serine (CHR; BDH, Eastleigh, Hants.) sufficient to double the serine concentration (*i.e.* to 38.06 mM) was added to test whether the culture was now serine-limited.

2.3 CELL ANALYSIS

2.3.1 Microscopic analyses of intracellular inclusions

The lipophilic stain Sudan Black (Burdon, 1946) was used to visualise intracellular granules for light microscopy. In addition unstained cells were examined using Normarski differential interference (DIC) microscopy.

2.3.2 Extraction and purification of polyhydroxybutyrate (PHB)

Polyhydroxybutyrate was extracted from washed cells by a modification of the method of Findlay and White (1983). Approximately 0.5 g of lyophilised cells were placed in a cellulose thimble (Whatman, UK) and extracted with chloroform for several hours in a Soxhlet apparatus (Tekator Ltd.). After cooling, the extract was transferred to a 100 ml flask and the chloroform was removed by rotary evaporation, leaving crude PHB as a film on the wall of the flask. The PHB was then purified by washing 4 times with 3 ml portions of ice cold ethanol, until the washings were colourless. This procedure was repeated with four 3 ml portions of cold diethyl ether, and the residue was dried by rotary evaporation, producing a white membrane-like solid. The material was dissolved in 25 ml of chloroform and the PHB concentration in 20 μl aliquots was determined by a modification of the method of Slepecky and Law (1960) as follows: 5 ml of concentrated sulphuric acid was added to the dry PHB in a screw cap tube and after thorough mixing the tubes were heated at 100°C for 30 min. This procedure dehydrated

the PHB to crotonic acid, the UV absorption of which was determined at 235 nm in silica cuvettes using a SP1800 spectrophotometer (Pye Unicam, Cambridge). A standard curve was constructed using PHB purified from a species of *Alcaligenes* (Sigma, Poole), determinations were done in triplicate and the standard deviation was never more than 3% of the mean.

2.3.3 Methanolysis and gas chromatography mass spectrometry of PHB

The purified material (0.5 mg) was dissolved in 1 ml chloroform in a teflon stoppered hydrolysis tube (Young's Scientific Glassware, Acton). The methanolysis reagent (1.9 ml), containing 1.7 ml methanol and 0.2 ml concentrated HCl, was added and the tube was flushed with nitrogen, sealed and placed in a heating block at 100°C. After 4 hours of methanolysis the tube was allowed to cool before the volume was reduced by approximately four-fold by evaporation in a stream of nitrogen. Deionised water (2 ml) was added and the mixture was extracted in chloroform. The chloroform layer was removed and analysed by gas chromatography mass spectrometry. Gas chromatography was performed using a Carlo-Erba 5160 "Mega" series chromatograph with a 25 m x 0.2 mm fused silica column coated with BP-1 (SGE Ltd., Milton Keynes). Helium was used as the carrier gas with a linear velocity of 30 cm s^{-1}. Samples were introduced by split injection at an injector temperature of 250°C. The gas chromatography oven temperature was programmed to rise from 50°C to 100°C at 5°C min^{-1}, and then to 200°C at 20°C min^{-1}. The column was coupled directly to the combined electron impact/chemical ionisation source of a Kratos MS80 mass spectrometer. Electron impact mass spectra were obtained at an ionisation energy of 70 eV and 100 μA trap current, and a source temperature of 200°C. The magnet was scanned downwards from 500 atomic mass units at a rate of 0.3 seconds per decade mass, with an interscan period of 0.2 seconds. Chemical ionisation spectra were obtained using isobutane as the reagent gas at a source pressure corresponding to an ion gauge reading of 10^{-4} torr, a beam current of 500 μA and a source temperature of 150°C.

2.3.4 ^{13}C Nuclear magnetic resonance (NMR) spectroscopy

Thirty micrograms of purified material was dissolved in 4 ml of deuteriochloroform in a 10 mm NMR tube. Proton decoupled NMR spectra were obtained with a Varian FT80 Fourier transform spectrometer, at ambient temperature and a field strength of 20 MHz. Twenty thousand transients were acquired using a pulse width of 21 μs (90° pulse), a 2 second acquisition time and a 2 second pulse delay. Fully coupled spectra were recorded by switching the decoupler off during acquisition and back on during the delay period (to maintain nuclear Overhauser effect and so improve sensitivity). For comparison a spectrum was also obtained for a commercially available preparation of PHB (produced by an *Alcaligenes* species and obtained from Sigma). This material was more soluble, so a solution of 70 mg in 3 ml of deuteriochloroform was used, and 1800 transients acquired. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane, using deuteriochloroform as the secondary reference.

2.3.5 Molecular mass estimation

The molecular mass of the purified material was estimated by non-aqueous gel filtration chromatography, using 2 mixed gel columns (Polymer Laboratories) in series, with chloroform as the mobile phase at a temperature of 40°C and a flow of 1 ml min^{-1}. The column was calibrated over the range 500 to 5 x 10^6 using polystyrene standards. The eluent was monitored by refractive index detection on a Polymer Laboratories data station. (I am grateful to Drs A. Webb and D. Byrom of ICI Billingham for these measurements.)

2.3.6 Fatty acid and polar lipid extraction

Cellular fatty acid samples were prepared as described by Wait (1988). Briefly, freeze dried cells were incubated overnight at 60°C with 2 ml of toluene/methanol (1:1), containing 50 μl concentrated sulphuric acid, in a 5 ml screw capped tube. The tubes were cooled, 1 ml of saturated salt solution was added, and the fatty acids extracted into hexane:chloroform (4:1). The extracts were dried in a stream of nitrogen, and

redissolved in 1ml trimethylpentane for gas chromatography.

Phospholipids were extracted by a modification of the procedure of Bligh and Dyer (1959). Approximately 2 mg of freeze-dried biomass was shaken with 1 ml of a monophasic mixture of chloroform, methanol and water (1:2:0.8). Additional water and chloroform (0.3 ml of each) were then added so as to force a phase separation. The organic layer containing the phospholipids was removed, washed with water, concentrated under nitrogen, and redissolved in 100 μl chloroform:methanol prior to analysis by positive and negative fast atom bombardment (FAB) mass-spectrometry.

The position of fatty acid substitutants in the phospholipids were determined by digestion with phospholipase A_2. The samples were dissolved in 3 ml of diethyl ether/methanol (98:2), and 10 units of *Crotalus atrox* phospholipase A_2 (Sigma, Poole) in 0.5 ml borate buffer (pH 8.9) were added. After incubation overnight at 37°C with constant shaking, the digestion products were recovered by Bligh-Dyer extraction and analysed by FAB-mass spectrometry.

2.3.7 Gas chromatography mass spectrometry of membrane polar lipids

Gas chromatography was performed with a Carlo-Erba 4130 chromatograph fitted with a 25 m x 0.2 mm fused silica column, coated with BP-5 (SGE limited) using helium as carrier gas at a linear velocity of 30 cm s^{-1}. The samples were introduced by splitless injection (splitless time 30 seconds) at an initial oven temperature of 80°C. After a one minute delay the oven temperature was programmed at 40°C min^{-1} to 205°C, and this temperature was held for 15 minutes. All peaks were identified by gas chromatography/mass spectrometry using a Kratos MS80 RFA mass spectrometer, interfaced to a Carlo-Erba 5160 chromatograph. Chromatographic conditions were as above, except that a 25M BP-1 (SGE Ltd.) column was used. The instrument was operated in electron-impact mode at an ionisation energy of 70 eV, trap current of 100 μamp, and a scan rate of 0.3 seconds per decade of mass. FAB mass spectra were

obtained with a Kratos MS80 RFA equipped with an Ion-Tech saddle field gun using xenon (8KeV) as the bombarding gas. The accelerating voltage was 4 KV and the magnet was scanned downwards from mass 1500 at 30 sec per decade of mass. One μl aliquots of the polar lipid extracts were applied to the stainless steel FAB target which had been previously coated with liquid matrix used (dithiothreitol/diethylerythritol (5:1) containing 1% 5-crown-15). Scans were acquired under the control of the DS90 data system which had been previously calibrated with caesium iodide clusters over the mass range of interest.

2.4 LETHAL DOSE 50 (LD_{50}) DETERMINATION

To assess the virulence of *L. pneumophila* grown under different conditions, samples of steady-state cultures were used as aerosol challenges for guinea-pigs as described by Baskerville *et al.* (1981) and Fitzgeorge *et al.* (1983). Culture samples were centrifuged and the cells resuspended in sterile distilled water (approximately 10^{10} bacteria ml^{-1}) and this suspension or serial dilutions (in sterile distilled water) of it were aerosolised using a three jet Collison spray at 65% relative humidity (RH) in a Henderson-type apparatus. Female Dunkin-Hartely guinea-pigs of Category 4 health status (MRC, 1974) weighing 300-350 g were exposed to this aerosol for 5 minutes. Standardisation of the bacterial suspensions used to create the aerosol was done by total count using a Helber counting chamber and dark background illumination. Quantification of the retained inhaled dose of bacteria was obtained by plating lung macerate, from animals which had received the highest dose, and killed immediately after challenge, on to BCYE agar and incubating at 37°C. Guinea pigs received either this dose or serial dilutions of it. In the second experiment the retained number of bacteria was measured at more than one dilution to ensure that the titration series was accurately reflected in the doses received. The number of responders and non-responders was recorded.

Preliminary experiments were carried out to obtain an approximate value for the

median lethal dose (LD_{50}) using 10-fold dilutions of *L. pneumophila* suspensions. For accurate determination of the LD_{50} values, the range of titrations used was reduced and 2.5-fold serial dilutions were used to give more points around the approximate LD_{50} values obtained in the preliminary experiments, this resulted in an interval of 0.4 after the counts had undergone a $\log_{10}$ transformation. Six doses of cultures grown at 37°C were used; but only four dilutions of cultures grown at 24°C were used, since the preliminary experiments indicated that *L. pneumophila* was avirulent after growth at this temperature. The number of subjects at each dose was increased from four to eight. The exact limits of the 95% confidence interval of the LD_{50} values were calculated using the method described by Finney (1964) for determining the fiducial limits of the LD_{50}. Heterogeneity in the data was allowed for by incorporating the modifications which Finney had described earlier (Finney, 1947).

Chemostat cultures of each strain were grown at various temperatures. To test the hypothesis that temperature had a reversible effect on the virulence of *L. pneumophila*, the culture temperature was cycled from 37°C to 24°C and back to 37°C.

2.5 ANALYSIS OF SUPERNATANT

2.5.1 Protein assay

The concentration of protein present in samples was determined using a commercially available dye-binding assay based on the differential colour change of an acidic solution of Coomassie Brilliant Blue G-250 in response to binding protein (Bradford, 1976) (Bio-Rad, Hemel Hempstead, Herts.). A calibration curve was constructed using bovine serum albumin.

2.5.2 Protease activity in cell free culture supernatant

The proteolytic activity present in the supernatants of *L. pneumophila* grown under different conditions was assayed for using the chromogenic protein derivative,

azocasein (sulphanilamide-azocasein; Sigma, Poole) as the substrate. Culture supernatants were filter-sterilised using 0.2 μm cellulose acetate (low protein binding) membranes (Sartorius, Gottingen, Germany). Azocasein stock solutions (20 mg ml^{-1}) were prepared by dissolving azocasein in 0.1 M Na_2HPO_4 buffer pH 7. This was clarified by centrifugation at 12000 x g for 10 minutes and filter-sterilised using a 0.2 μm cellulose acetate membrane (Sartorius, Gottingen, Germany). Substrate and supernatants were equilibrated at 37°C. Aliquots of substrate (250 μl) were added to sterile microcentrifuge tubes. The reaction was initiated by adding 250 μl of supernatant into the substrate. The digests were mixed gently and incubated at 37° C for 1 hour. The assay was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) to each tube. Blanks were prepared by incubating azocasein and culture supernatants separately for 1 hour at 37° C before adding 250 μl of supernatant to a microcentrifuge tube, followed by 1 ml 10% (w/v) TCA and then 250 μl of substrate. After being allowed to stand for 15 minutes (to ensure complete precipitation) the tubes were centrifuged at 13000 x g for 5 minutes. One ml of the supernatant from each digest was transferred into cuvettes (1 cm light path) containing 1 ml of 1.0 M NaOH. The absorbance was determined at 440 nm against the appropriate blank on a SP500 spectrophotometer (Pye Unicam, Cambridge). One unit of protease activity was defined as the amount of enzyme required to cause an absorbance change of 0.001 in a cuvette with a 1 cm light path, under the conditions of the assay.

2.6 INVESTIGATION OF IRON-LIMITED GROWTH

2.6.1 Low iron medium

In this part of the study ABCD broth was modified to cause iron restriction of the culture. Both ferrous sulphate ($FeSO_4.7H_2O$) and haemin were omitted from the medium, this new medium was named MOD2. The concentrations of all the other medium components remained the same. The concentration of iron in ABCD broth was

measured to be on average 160 μM and that of MOD2 to be between 0.3 and 0.5 μM. This residual iron was probably due to impurities in other medium constituents.

2.6.2 Culture

This part of the study was carried out using strain Corby grown at 37°C with the culture conditions as described in Section 2.1.4. When steady-state growth had been attained the virulence of the culture was measured by aseptically withdrawing samples of culture through the sample port. These were used to challenge guinea pigs as described previously (section 2.4). Since numbers of bacteria present in iron-limited cultures were too low to provide a similar concentration of cells to that in iron replete cultures it was necessary to centrifuge 100 ml of culture sample and resuspend it in 10 ml of sterile distilled water. Because the removal of such large volumes directly from the chemostat would significantly disturb the dilution rate it was necessary to allow time for the steady state to re-establish before subsequent sampling.

To investigate the iron requirement of strain Corby under these growth conditions ferrous sulphate solution was titrated into the medium reservoir incrementally, increasing the concentration of iron in the medium from 0.3 μM to 25 μM, and allowing time for steady-states to establish between the increases in iron concentration before further supplementation. Changes in biomass concentration were monitored as in Section 2.1.6. All glassware and centrifuge tubes used for collection and storage of iron-limited culture were acid washed in 6 M hydrochloric acid overnight and rinsed in distilled deionised water.

2.6.3 Siderophore assays

The universal chemical assay for siderophores developed by Schwyn and Neilands (1987) was used to detected siderophore activity in filter-sterilised [using 0.2 μm cellulose acetate membranes (Sartorius, Gottingen, Germany)] culture supernatants of the *L. pneumophila* strain Corby grown iron replete at 24 and 37°C, and iron limited at

37°C.

2.6.3a *Preparation of Chrome azurol S (CAS) assay solution*

Hexadecyltrimethylammonium bromide (HDTMA; 6ml) was placed in a 100 ml volumetric flask and diluted with water. $FeCl_3.6H_2O$ (1.5 ml; 1.0 M; Sigma, Poole), in 10 mM HCl was mixed with 7.5 ml of 2 mM aqueous CAS (Sigma, Poole) solution, and the mixture was slowly added to the flask with stirring. Anhydrous piperazine (4.307 g) was dissolved in water and 6.25 ml of 12 M HCl and rinsed into the flask. The volume was made up to 100 ml with water. All glassware was washed with 6 M HCl and double distilled water (produced in an acid washed glass still) was used throughout these experiments.

2.6.3b *Assay procedure*

Culture supernatant (0.5 ml) was mixed with CAS assay solution. A reference was prepared using uninoculated medium: ABCD broth in the case of iron replete samples and MOD2 when the culture was iron limited. The absorbance of the mixture was measured at 630 nm in a UV-260 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) reading against either ABCD or MOD2 as appropriate. The determinations were done in triplicate when the reactions had reached equilibrium. The standard deviations were never more than 7% of the means.

2.6.3c *Standard curve*

A calibration curve was constructed using Desferal mesylate (desferoxamine methanesulphonate; DFOM; Ciba-Geigy Pharmaceuticals, Horsham). Aliquots (0.5 ml) of known concentrations of DFOM (over the range 0-20 μM) were mixed with CAS assay solution. In this instance the reference was prepared using water in place of the siderophore. The assay was blanked against water. Again the determinations were done in triplicate and the standard deviations were always less than 10% of the mean. With DFOM equilibrium was reached after 22 hours.

2.7 BIOLOG SUBSTRATE UTILISATION TEST

The BIOLOG bacterial identification system is based on substrate utilisation and employs a redox dye, tetrazolium violet, as an indicator of substrate utilisation. The panel of 95 test substrates is presented in a microtitre plate. Bacterial metabolism of the test substrate results in the formation of NADH which, in order to be reoxidised, passes on electrons to an electron transport chain. The tetrazolium dye is of suitable redox potential to accept these electrons and is irreversibly reduced to a purple formazan. The dye functions independently of the specific structure of the electron transport chain and is therefore of general application. It is thus able to detect the ability to metabolise any substrate, including amino acids which act as carbon and energy sources for the *Legionellaceae* (Pine *et al.*, 1979). The protocol described by the manufacturer was modified in several ways to facilitate its use with legionellae, and the modified procedure optimised.

2.7.1 Strains and cultivation

The strains used were all laboratory stocks which were maintained on glass beads at -70°C. They were all originally obtained from the UK National Collection of Type Cultures, Colindale, London. The cultures used were single strains of *L. pneumophila* serogroups 1-14 (excluding 4 and 9) and eight type strains of other *Legionella* species. Namely *L. bozemanii* (NCTC 11368), *L. dumoffii* (NCTC 11370), *L. feeleii* (NCTC 12022), *L. hackeliae* serogroup 1 (NCTC 11979), *L. israelensis* (NCTC 12010), *L. longbeachae* serogroup 1 (NCTC 11477), *L. micdadei* (NCTC 11371), *L. pneumophila* serogroup 1 (NCTC 11192), 2 (NCTC 11230), 3 (NCTC 11232), 5 (NCTC 11405), 6 (NCTC 11287), 7 (NCTC 11984), 8 (NCTC 11985), 10 (NCTC 12000), 11 (NCTC 12179), 12 (NCTC 12180), 13 (NCTC 12181) and 14 (NCTC 12174) and *L. rubrilucens* (NCTC 11987).

Prior to inoculation of the BIOLOG plate all isolates were grown on BCYE agar

(Edelstein, 1981) at 37°C for 72 hours. Since the BIOLOG system requires metabolically active cells it is considered necessary to use as young cultures as possible. It was possible to get sufficient growth on BCYE plates after 24 hours, if they were inoculated heavily from a primary plate itself inoculated from the freezer stock. When these were used to inoculate the BIOLOG plates it was found that slightly more substrates were utilised. However, it was difficult to obtain sufficient growth for some of the strains particularly those not belonging to *L. pneumophila*; in some instances the growth on three BCYE plates were barely adequate to provide a sufficient inoculum, especially when the higher cell concentration (described later) was used. Since it appeared that there was little to gain by this extra step, and to allow the development of a standard protocol (with a common incubation time) for the identification of all *Legionella* species. Buffered Charcoal Yeast Extract plates were incubated at 37°C for 72 hours to provide inocula for the identification of legionellae.

2.7.2 BIOLOG inoculation and incubation

The procedure described in the manufacturer's manual requires bacteria to be grown on agar medium, removed by rolling a sterile swab across the bacterial growth and suspending the bacteria in 0.85% (w/v) NaCl solution, taking care to avoid nutrient carry over from the medium. The protocol requires a cell density within a specified turbidity range set using the turbidity standards supplied by BIOLOG at that time, which was equivalent to an absorbance of 0.5 to 0.65 at 590 nm as measured on a SP 500 spectrophotometer (Pye Unicam, Cambridge, UK). (N.B. These turbidity standards are no longer recommended by BIOLOG since they were found to lack consistency. The standards now supplied by BIOLOG have an absorbance of 0.34 to 0.39 at 590 nm). In an effort to use a uniform cell density throughout the experiments an inoculum in the range of 0.55 to 0.6 was used (approximately 1.3-1.7 x 10^9 CFU.ml^{-1}). Page's amoebal saline (PAS; Page, 1967) was used in place of 0.85% (w/v) NaCl solution, as this concentration of NaCl has been reported to inhibit growth of several legionellae strains (Barbaree *et al.*, 1983). Bacterial suspensions used to inoculate the BIOLOG

plates were streaked on to BCYE minus cysteine agar to test purity since legionellae cannot grow without cysteine. The BIOLOG multiwell plates were inoculated with 150 μl of bacterial suspension as described in the BIOLOG User's Manual (Biolog Inc., Hayward, California) using a multichannel automatic pipette. Multiwell plates were incubated at 37°C in either air or a low oxygen atmosphere, since it is known that legionellae are sensitive to low levels of hydrogen peroxide and superoxide radicals (Locksley *et al.*, 1982; Hoffman *et al.*, 1983). The low oxygen atmosphere was obtained by evacuating an anaerobic jar, bleeding in 0.2 bar of air and filling the remaining volume with an anaerobic gas mixture (95% N_2: 5% CO_2).

2.7.3 Metabolic profiles and database comparison

A purple formazan formed in those wells where substrate was metabolised and the colour development in the multiwell plates was read by eye at 24 hour intervals up to 72 hours. Alternatively, the multiwell plates were read automatically in a Metertech Microplate Reader (Atlas Bioscan, Bognor Regis) with a filter cut-off of 600 nm. The 95 substrate wells were read against a substrate blank well. The BIOLOG manufacturer recommends for a positive result a minimum reading of 40% of the highest positive substrate response but experience lead to selecting a minimum reading of 20%. The substrate utilisation profile of each strain was recorded and used to construct a specific legionella database. The files in this database were compared against, and used to identify, subsequent known strains and replicate experiments of the same previously identified strains. The data were also compared against the currently available BIOLOG database of environmental and medical bacteria. Additional software was written in the laboratory which generated spreadsheets allowing easier comparisons of substrate utilisation between strains.

2.7.4 Testing of sensitivity and specificity

Recent isolates of legionellae (kindly provided by Dr. J. Kurtz, Oxford) were put through the modified BIOLOG procedure to test the efficiency of the system at

identifying non-type strains. The specificity of the modified procedure was evaluated by identifying environmental isolates of *Pseudomonas fluorescens, P. vesicularis, Sphingomonas paucimobilis, Klebsiella* spp., *Alcaligenes* sp., *Achromobacter* sp. and other water isolates with it.

2.7.5 Effect of increased biomass on metabolism

One possible means of encouraging growth and metabolism of microaerophilic bacteria is to increase their cell mass so that the higher respiration rate helps maintain a lower oxygen concentration diffusing into static cultures. To investigate the effect of increased biomass concentration on the aerobic metabolism of legionellae in the BIOLOG plates the concentration of the inoculum was doubled (approximately 2.3-3.0 x 10^9 CFU ml^{-1}) and the multiwell plates incubated in air.

2.7.6 Simplification of diluent

The effect of replacing PAS with deionised water as diluent was investigated in an attempt to simplify the modified protocol.

2.8 DESIGN OF MICROBIOLOGICALLY CONTAINED EVAPORATIVE COOLING TOWER AND ITS ASSOCIATED SYSTEMS

2.8.1 Evaporative cooling tower

An evaporative cooling tower and heat exchanger were constructed from galvanised steel, a material commonly used in the construction of cooling towers (see Figure 2.1 and Plate 2.3). The cross-sectional area of the tower was 0.3 m and the height 1.2 m. The tower was of the contra-flow induced draught configuration with a variable speed fan providing air movement and was comprised of the components found in real systems, namely: pond; pack; water distribution system; drift eliminator; and fan (Plate 2.4).

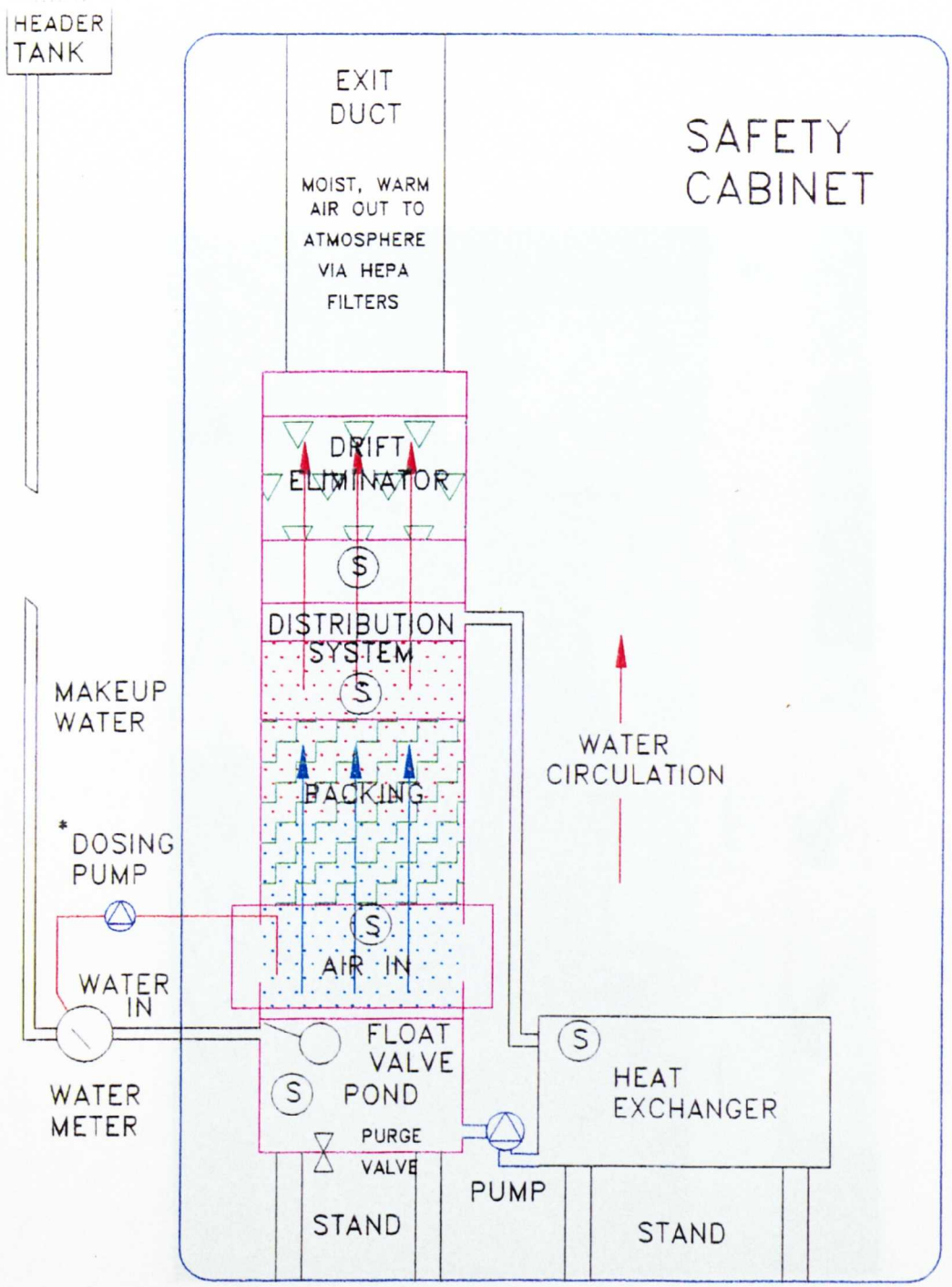


Figure 2.1.

Simplified view (front) of cooling tower
& microbiological safety cabinet
Stuart Mauchline June 1994

(S) Biofilm sampling point.

* Dosing pump is driven by an electrical pulse from the water meter.

Tower internal cross section :- 300 X 300mm.
Overall tower height (inc. pond) 1230mm.
Tower is housed in microbiological safety cabinet, dimensions (W X H X D) 1200 X 1960 X 800mm.
HEPA filter housing connects to the exit duct via a transformation piece.

Plate 2.3 Cooling tower.

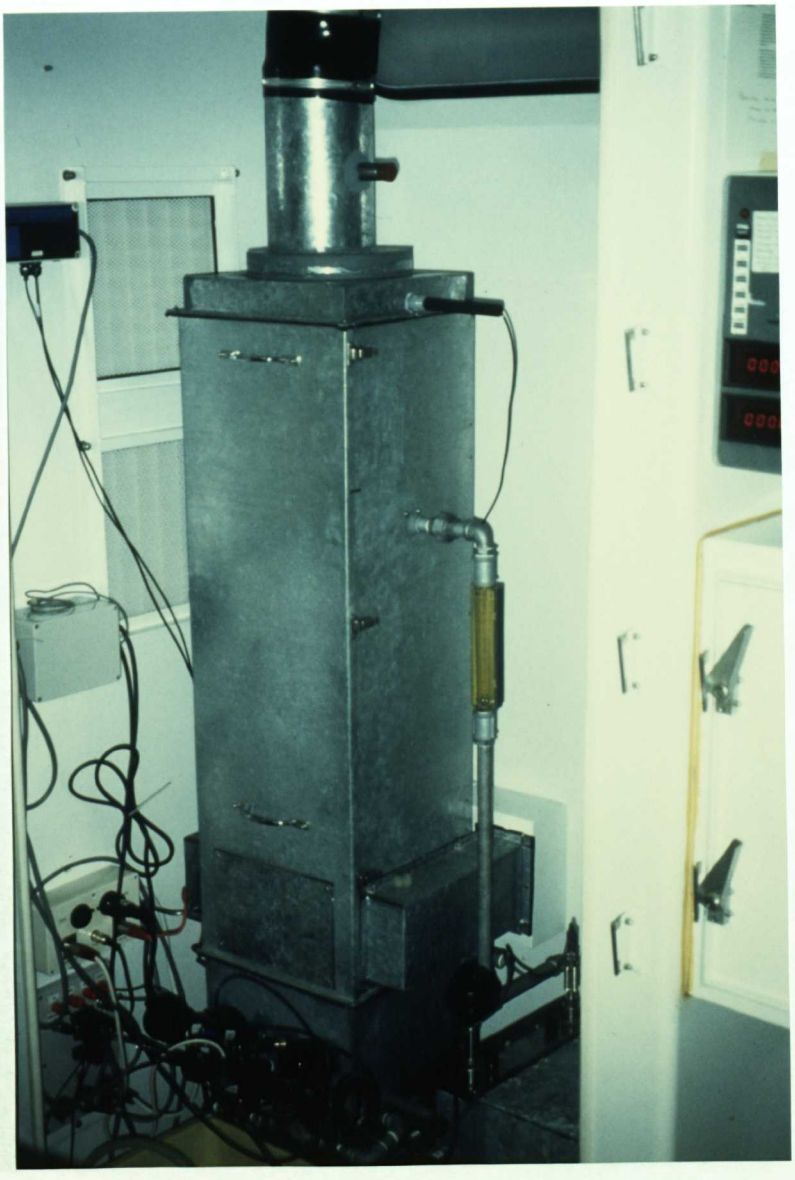
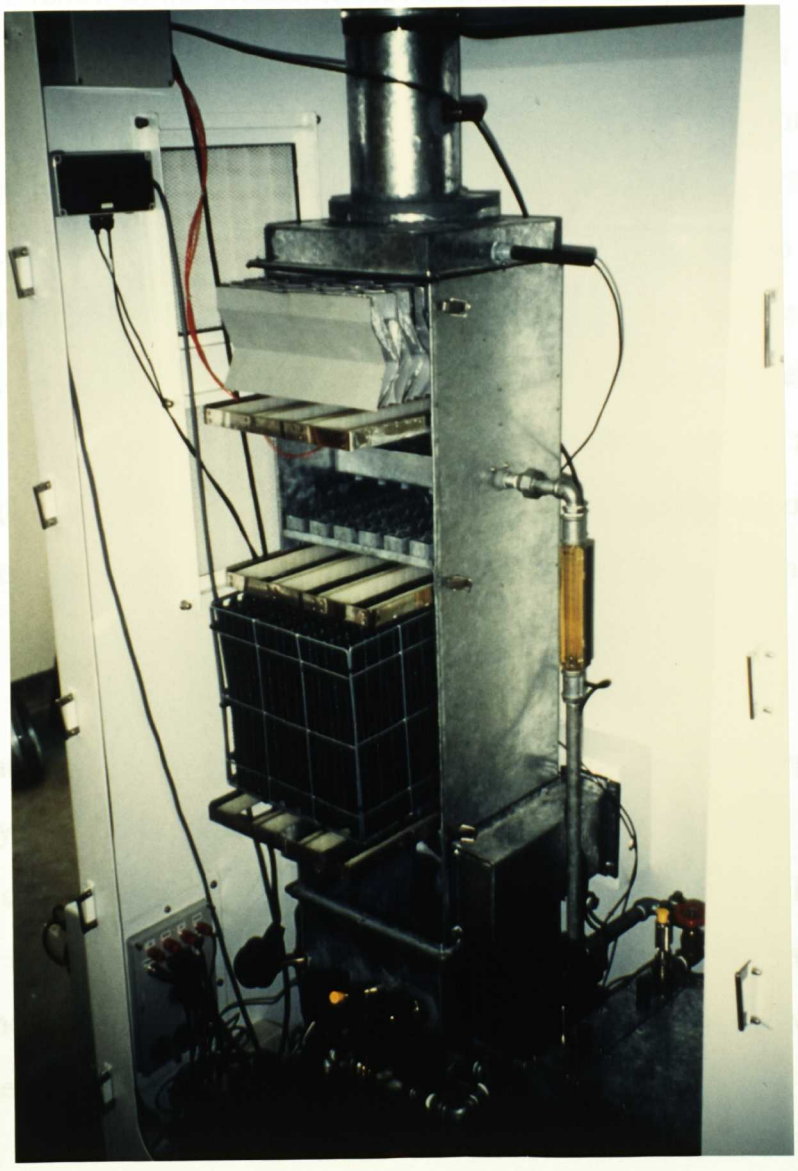


Plate 2.4 Cooling tower with front panel removed, demonstrating its components.



2.8.1a *Pond and make-up water (see Figure 2.1 and Plate 2.4)*

The pond (or cold water basin) received make-up water replacing water loss due to evaporation, drift, purge and leakage and is also the point at which cooled water is collected before return to the process requiring cooling. Water, stored in a dedicated break tank, was supplied to the cooling tower pond, via an impulse water meter which provided an electrical pulse to a Gamma 4I/1000PP dosing pump (Prominent Fluid Controls (UK) Ltd, Swadlincote, Derbyshire) to add Polymate C92/81 scale and corrosion inhibitor (Grace Dearborn, Widnes, Cheshire) in proportion to the make-up water. The break tank was separated from the building main cold water supply, for safety reasons, by an air break and a double check valve. The water level in the pond was controlled by a float valve. It was, however, not possible to include an over-flow, therefore a high level switch was installed to provide protection against over-filling of the pond. The switch, when activated, closed a solenoid valve on the make-up water supply.

The concentration of total dissolved solids (TDS) in the recirculating water was determined by measuring the conductivity using an IN2-10-T10K temperature compensated conductivity cell [Automated Water and Effluent (AWE), Stafford] connected to an Aquatronic conductivity controller from the same supplier. The conductivity meter provided a control output to operate a solenoid purge valve when a pre-programmed set point was exceeded.

2.8.1b *Pack, distribution system and drift eliminators (see Figure 2.1 and Plate 2.4)*

The pack, which maximises water cooling, was standard plastic packing material (Carter Industrial Products Ltd, Birmingham). The distribution system, used to distribute water from the pond evenly over the pack, was of the trough and gutter type. A double pass drift eliminator (Marley, Worcester), to reduce escape of water droplets into the air, was located above the distribution system.

The heat/cooling load was supplied by a modified W32 water bath (Grant, Cambridge) which had three 1 kW heating elements integrated into it. Taken together with the 1.4 kW heater included in the water bath this gave a total heating capacity of 4.4 kW. Water from the modified water bath was circulated to the heat exchanger jacket by a magnetically coupled water pump (RS Components, Corby, Northants.). Panacide M (Coalite Chemicals, Bolsover, Derbyshire) was used to control microbial growth in the heat load water.

2.8.2 Containment

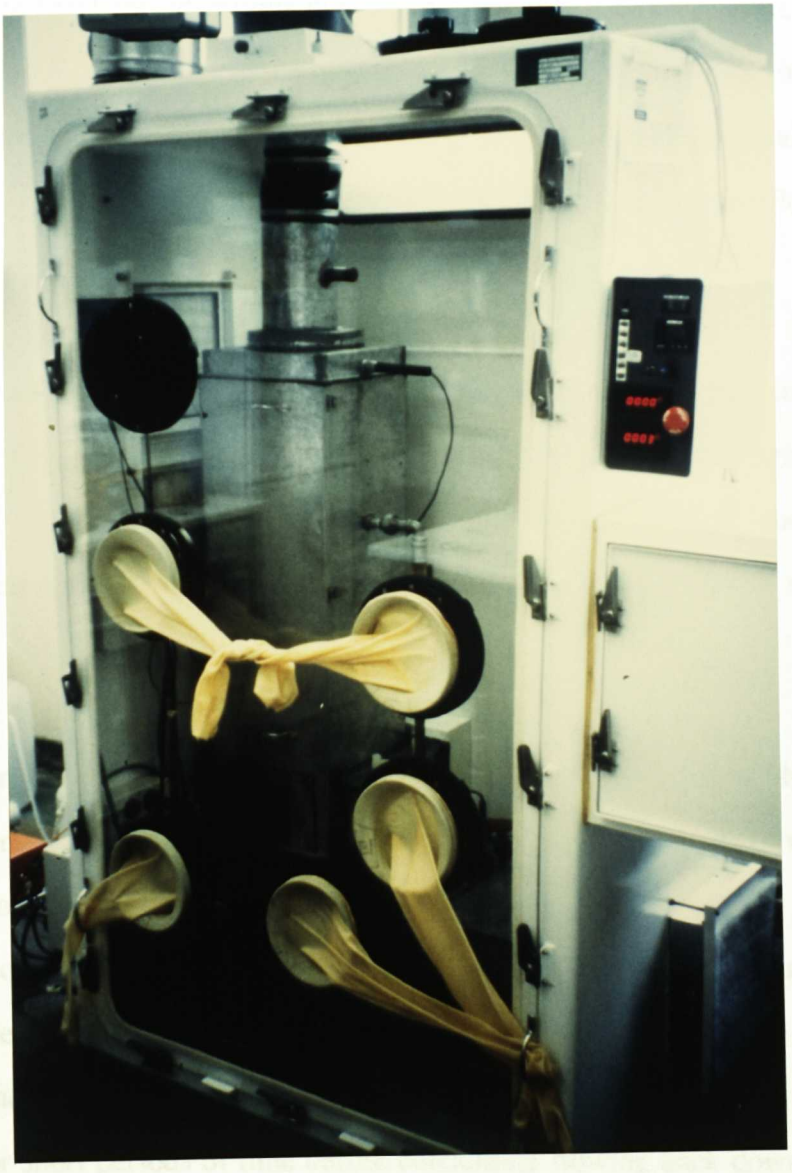
In order to seed the system with pathogenic legionellae it was necessary to provide microbiological containment. A class III microbiological safety cabinet was specially constructed to house the cooling tower and heat exchanger (Figure 2.1, Plate 2.5).

Large volumes of unfiltered air from outside the building were supplied to the cooling tower to accurately reflect the natural air scrubbing carried out by "commercial" cooling towers. This was achieved by ducting air from an intake on the roof of the building to the tower. An air-tight damper was fitted in the intake duct and back-draught shutters installed in both the intake and exit ducts to prevent contaminated air flowing back along the intake duct. The air-tight damper was only open while there was an inward movement of air (detected by a pressure sensor). If the movement of air from the intake to exit direction was insufficient, or if there was a power failure, the damper would close under spring-action, thus providing a fail-safe mechanism. Air exiting the tower was passed through a double high efficency particulate air (HEPA) filter before being vented to atmosphere.

2.8.3 Air supply

A number of difficulties had to be overcome in the setting up of the tower. One of the most formidable problems encountered was providing up to 750,000 litres of outside air per hour to the tower and then venting moisture laden air to atmosphere.

Plate 2.5 Cooling tower inside Class III microbiological safety cabinet.



2.8.3a *Original design*

When the project was first conceived it was envisaged that the air inlet and exit would be situated on the window of the laboratory to minimise the runs of ducting required. However, due to local considerations this was not possible. The air-intake and exit was therefore sited on the roof. The laboratory where the cooling tower was situated was on the first floor of a two storey building with a service floor immediately above the first floor. Ducting was run from the intake (Plate 2.6) through the service floor and into the laboratory.

When the tower was first installed a small axial fan, chosen subsequent to expert ventilation advice, was sited on the top of the cabinet to pull air through the cooling tower (Plate 2.7). However, due to the change in configuration of the ducting and the changes this caused to the air flow, the fan was significantly under sized for its purpose.

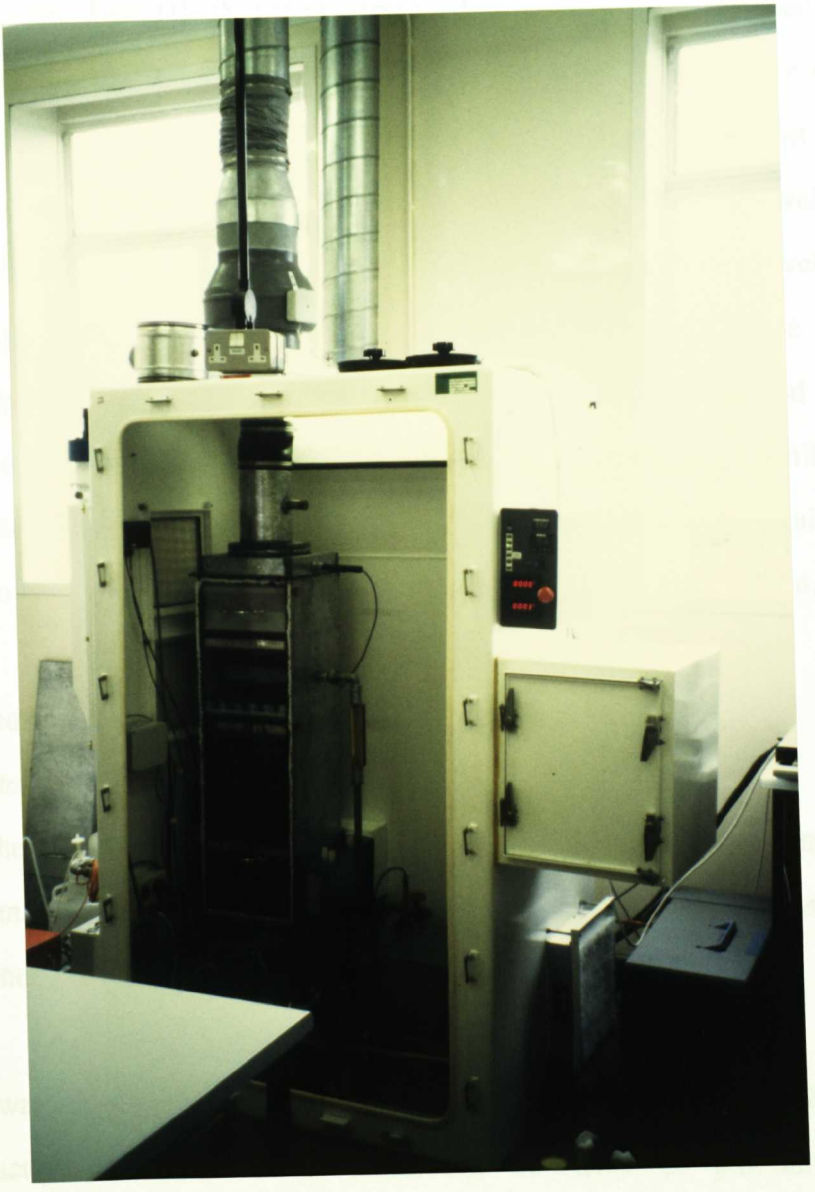
A HEPA filter was installed between the roof of the cabinet and the fan. This arrangement proved to be unsatisfactory for two reasons. The first, was that the configuration of the inlet ducting, components of the tower and the HEPA filter restricted air flow. The achievable air velocity was 0.5 m s^{-1} which was not sufficient to simulate the air flows encountered in operational cooling towers. The second problem was the build up of condensation in the air outlet ducting. The tower could only operate for short periods of time before condensate flowing back down the vertical exit ducting compromised the HEPA filter and the fan. Nevertheless, it was possible to carry out some experiments at low air speed using the cooling tower in this format. However, urgent modifications were required to facilitate subsequent work.

2.8.3b *Air supply modifications*

To facilitate improved air flow three key modifications were made. Firstly, a three phase air conditioning fan with much greater flow capacity was installed on the roof

Plate 2.6 View of roof showing the cooling tower fan (after modification) and the air intake.

Plate 2.7 Cooling tower and safety cabinet with original tower fan installed.



(Plate 2.6). This fan had stepped speed control allowing variation of air flow. Secondly, larger surface area HEPA filters were installed to cater for the increased through-put of air. Two HEPA filters, arranged in series, were installed in the exit duct. The cross-sectional surface area of the HEPA filters was greater than that of cooling tower, therefore, a transformation piece was installed to direct air flow across the full surface area of the filters (Plate 2.8). This reduced the face velocity at the filters to within a safe operating range while allowing the required air velocity in the tower. Although safety guidelines only require passage through a single HEPA filter before venting to atmosphere it was considered prudent to exceed this safety requirement because the filters would be subject to constant challenge whilst the tower was in operation. Thirdly, to alleviate the problem of condensation in the air outlet duct a condensation trap was included and the duct was lagged and trace-heated.

2.8.4 Data acquisition and logging (Plates 2.9 and 2.10)

2.8.4a *Monitoring instrumentation*

To monitor the environment in the cooling tower a comprehensive computer based logging system was installed, this included a series of immersion probes in both the pond and the heat challenged/warm side of the heat exchanger.

Temperature was measured using TPAI-N temperature probes (AWE, Stafford), which were constructed of 316 stainless steel and consisted of platinum resistance thermometers, connected to a digital panel meter (ITT Instruments, Slough, Berks.) via temperature transmitters (RS Components, Corby, Northants.).

pH was measured using temperature compensated, gel filled pH probes (AWE, Stafford) and a pH controller (B & C Electronics, Carnate, Italy).

Redox potential was measured using platinum sealed combination redox electrodes (AWE, Stafford) connected to a mV controller (B & C Electronics, Carnate, Italy).

Plate 2.8 Cooling tower installation with large surface area HEPA filters.

Plate 2.9 Probes in the pond and heat exchanger.

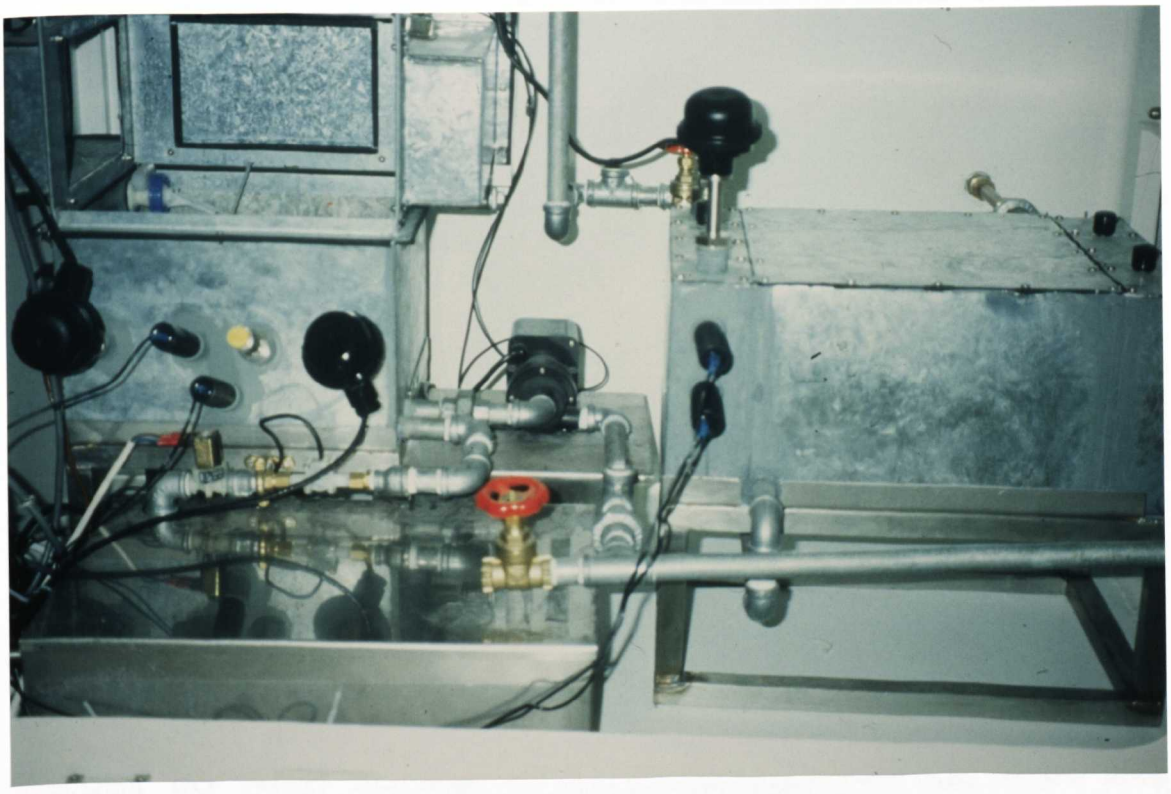
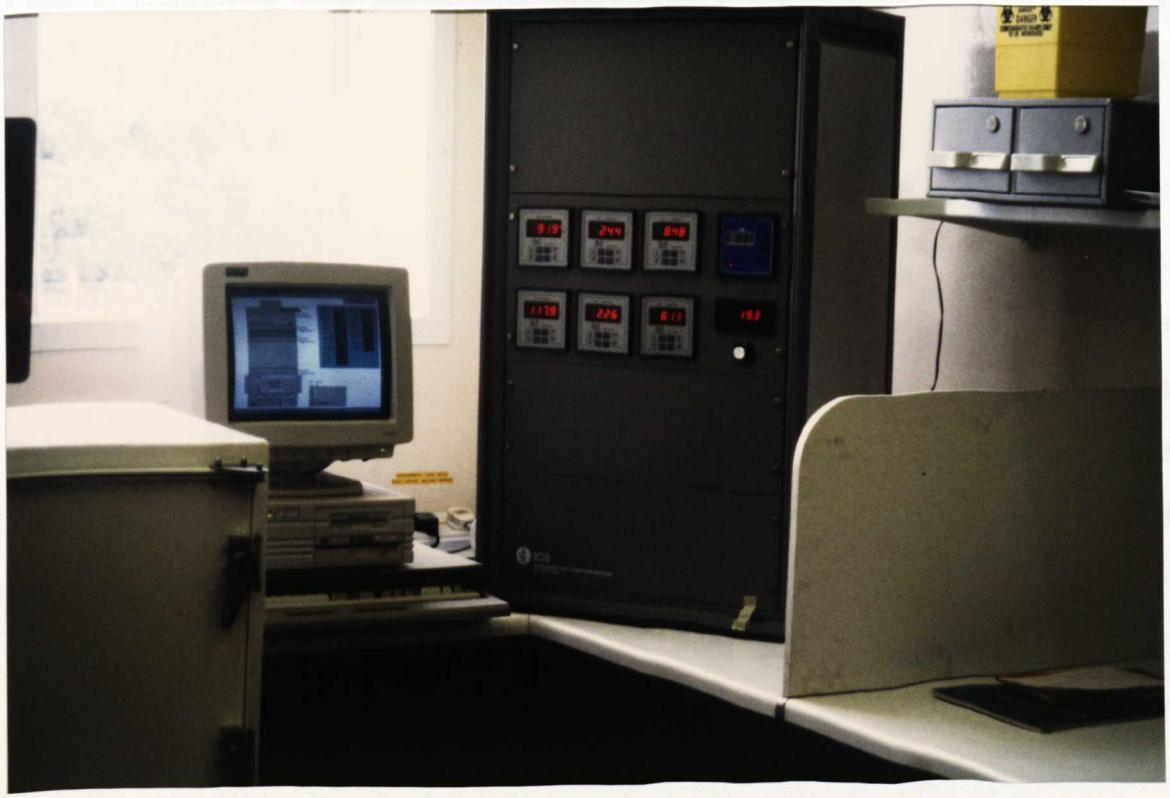


Plate 2.10 Instrument cabinet and data logging computer.



The dissolved oxygen concentration was measured with Ingold polarographic oxygen electrodes (Mettler-Toledo Ltd, Leicester) and OD 7615 meter (B & C Electronics, Carnate, Italy), temperature compensation was provided by a Pt100 sensor.

Air speed in the tower and intake air temperature were measured using a 129T hotwire anemometer and a 385T probe connected to a 900I/V transmitter (Solomat, Bishop's Stortford, Herts.).

The conductivity was monitored in the pond as described previously (see Section 2.8.1a).

2.8.4b Logging system

Analogue signals from meters and transmitters were conditioned using a T100 Intelligent I/O system (Turnbull Control Systems Ltd, Worthing) before being transmitted to the customised logging program (Biotechnology Computer Systems, London) resident on a M-333 personal computer (Elonex, London). Monitoring was carried out on a 3 second loop and values were logged every hour (values used for logging were averaged over 6 seconds to ensure a representative reading).

The volume of make-up water, measured using a flow meter, and the purge volume, measured by collecting all the waste water from the cooling tower, were logged onto the computer manually. Air dry and wet bulb temperatures were measured using a whirling hydrometer (Gallenkamp). The wet bulb temperature and calculated relative humidity were entered to the data logging program. Pump and fan activities were also recorded on the logging software.

2.8.5 Biofilm sampling points

Biofilm sampling points were sited at the heat exchanger, the pond, below and above the packing, and between the distribution system and the drift eliminator (Figure 2.1).

2.8.5a Original biofilm sampling design

When the cooling tower was first designed the intention was to collect biofilm from slides (100 mm x 76 mm) held in racks, which would slot into positions in the body of the tower, suspended in the pond, or be held in a rack welded onto the underside of inspection plates on the manifolds of the heat exchanger.

The panel making up the front elevation of the tower above the level of the pond was held in place by clips with neoprene foam (RS Components, Corby, Northants.) providing a seal around its edge. It was designed to be removed to gain access to the slide racks in the pond and the body of the tower body, allowing recovery of biofilm samples from the slide racks positioned immediately below the drift eliminator, above and below the pack and from the pond. The biofilm sampling by this method was physically difficult, given the movement restrictions imposed by operating under microbiological containment; it was not possible to routinely remove and replace the slides aseptically. In addition, the panel seal did not recover after opening and re-closing resulting in leakage of water from the cooling tower. This was a significant problem given that any water leaking from the system would build up inside the safety cabinet.

To collect biofilm samples from the heat exchanger it was necessary to unscrew the inspection plates on the heat exchanger and extract the slide racks. This also presented some difficulties: firstly the act of unfastening and refastening ten screws, some quite inaccessible, proved to be awkward and time consuming; and similar difficulties were encountered in the cooling tower, in aseptically recovering slides and resealing the equipment after sample collection. It was not possible, therefore, to collect biofilm

samples satisfactorily until suitable modifications could be effected.

2.8.5b Biofilm sampling modifications

To avoid removing the front panel of the tower several new openings were engineered into this panel. Access to the cooling tower pond was made through a door on the front of the tower and two slide holders were hung from clips immediately inside this opening. Three sampling points, consisting of stainless steel racks which gained access to the body of the tower via its front wall, were situated at points above the tower pond. Duplicate slides were placed at each sample point. The inspection plates on the heat exchanger were re-engineered to increase their rigidity and new sample points were included allowing the inspection plate to remain in place during normal operation.

The heat exchanger sample points consisted of: two ports welded into the heat challenged/warm-side manifold of the heat exchanger, a blinding plug with O'-ring seal fitted into each and was held in place by a knurled locking nut (this gave the required pressure resistance). A stainless steel extension was welded onto the blinding plug, holes were drilled at the distal end of this extension and this acted as the point of attachment of sampling slides. Weld-in ports and sealing plugs were obtained from Mettler Toledo Ltd. (Leicester). Sampling slides were tied in place on the heat exchanger and pond with titanium wire, other slides were slotted into locators on the slide racks.

2.9 EXPERIMENTAL DESIGN OF MICROBIAL COLONISATION OF THE EVAPORATIVE COOLING TOWER

2.9.1 Inoculum for cooling tower

Microorganisms entered the cooling tower in the make-up water and from the air stream, similar to commercial cooling towers. In addition, the tower was inoculated with a naturally grown population of infectious *L. pneumophila* serogroup 1

(monoclonal subgroup Benidorm; restriction fragment length polymorphism [RFLP] type 14) and its supporting microbial flora. This consortium had originally been obtained from a water system responsible for an outbreak of Legionnaires' disease (Colville *et al.*, 1993) and was maintained by routinely replacing a proportion of water with fresh tap water, but without subculturing on artificial media which might affect the phenotypic and genotypic expression of the organisms and thus alter their behaviour in the environment (Kuchta *et al.*, 1985). Aliquots of this microcosm were used to seed the system to give a final concentration of 1000 to 2000 CFU l^{-1} of *L. pneumophila* in the circulating water. This concentration was considered to be a realistic concentration of legionella contamination on the assumption that when a cooling tower becomes colonised with legionellae, only low concentrations of the pathogen enter the system. Immediately after inoculation the pump was run for 20 minutes (approximately 5 volume circulations) to allow adequate mixing before an initial sample was collected.

2.9.2 Water analysis

The physicochemical parameters of the water from the evaporative cooling tower were analysed. Briefly this was carried out as follows. Water samples were sterilised by passage through 0.2 μm cellulose acetate membranes prior to analysis. Concentrations of metals, ammonia and phosphate in water samples were measured as described below.

Sodium and potassium were determined by flame emission spectroscopy using a Philips PU9100X spectrometer and an air-acetylene flame, with detection at 589.0 nm and 766.5 nm respectively.

Calcium, copper, lead, magnesium, manganese and zinc were measured by atomic absorption spectroscopy using a Philips PU9100X spectrometer and an air-acetylene flame. The detection wavelengths were 422.7 nm, 324.8 nm, 217.0 nm, 285.2 nm, 279.5 nm and 213.9 respectively.

Aluminium and molybdenum were determined as above but with the substitution of a nitrous oxide-acetylene flame. Detection wavelengths of these metals were at 309.3 nm and 313.3 nm respectively.

Iron concentrations were determined by electrothermal atomic absorption spectroscopy using a Philips PU9100X spectrometer and electrolytic graphite cuvettes, with detection at 248.3 nm this gave enhanced sensitivity. This facility was not available during the initial cooling tower experimental run. (During Modes 1 and 2 iron concentration was determined by flame atomic absorption spectroscopy.)

Ammonium concentration was established by colorimetric analysis involving the Berthelot reaction as described by Gordon *et al.* (1978).

Chloride, phosphate and sulphate were quantified by High Performance Ion Chromatography using a Dionex AS4 ion-exchange column and conductivity detection after membrane-suppression of eluant buffer.

Silicate was determined colorimetrically by formation of a molybdenum blue complex (Vogel, 1983).

Total solids (TS) were determined gravimetrically.

Total organic carbon (TOC) was determined by infra-red detection of carbon dioxide following oxidation by persulphate and ultra-violet radiation (Clesceri *et al.*, 1989) using a Protoc analyser (Pollution Process Monitoring, Sevenoaks, Kent).

Hardness was calculated from calcium and magnesium values as equivalent calcium carbonate (Clesceri *et al.*, 1989).

TOC and hardness analyses were only available towards the end of the project.

2.10 MICROBIOLOGICAL TESTING OF COOLING TOWER

2.10.1 Water samples

Samples of water were collected from the purge valve after an initial pre-flush to remove bacteria which may have been trapped in the purge line. To minimise the disruption to normal operation samples were collected, whenever possible, during the routine purge from the system which is activated by the conductivity controller.

2.10.1a Detection of Legionella

When the concentration of legionellae was low, counts were obtained by concentrating legionellae density in the sample by filtration. This was carried out by vacuum filtering 500 ml of water through a 47 mm diameter, pore size 0.2 μm, cellulose nitrate membrane (Sartorius, Gottening, Germany) and resuspending the filtrate in Page's Amoebal Saline (PAS; Page, 1967). When *L. pneumophila* numbers were high the concentration procedure was omitted. Aliquots of 0.1 ml of the neat or concentrated sample were plated directly onto legionella selective agar: Buffered Charcoal Yeast Extract plus glycine, vancomycin, polymyxin and cycloheximide (GVPC) agar (Dennis *et al.*, 1984). In addition, a portion of the sample or concentrated sample was heated at 50°C for 30 minutes before plating on to GVPC. The inoculated plates were incubated at 37°C for 12 days with plates being inspected daily. Counts were carried out in duplicate. Putative *Legionella* isolates grown on GVPC were examined with a plate stereo-microscope and confirmed by sub-culturing onto Buffered Charcoal Yeast Extract (BCYE) agar and on to BCYE agar deficient in L-cysteine (NCM) agar, following absence of growth on the latter medium. Serology was used to confirm that the legionellae isolated were *L. pneumophila* of the same type as the original inoculum (see section 2.10.3 a).

2.10.1b Detection of heterotrophs

Isolation and enumeration of heterotrophic bacteria was carried out by serially diluting volumes of cooling tower water in sterile PAS to give plate counts in the region of 30 to 300 CFU per plate. Aliquots of 10 or 100 μl were spread onto R2A agar, a low nutrient medium designed for isolation of bacteria from water (Reasoner & Geldreich, 1985). Plates were incubated at 22°C for 7 days before counts and descriptions of resultant colonies were made. Counts were carried out in duplicate.

2.10.2 Sampling of biofilm from cooling tower

Slides were added and removed and the titanium wire tied using sterilised scissor clamps (RS Components, Corby, Northants.) and bone-cutters (Rocket Instruments, London). Sterile galvanised steel slides (30 mm x 10 mm) were placed at the locations described in Section 2.1.5. Two slides were situated at each location, one was removed weekly and replaced with a sterile replacement; the other remained in place until the end of a mode of operation. Sampling was effected by removing the slides with sterilised instruments and submersing them in 10 ml of sterile PAS.

Surfaces of the slides were scraped with sterile plastic cell scrapers (Nunc, Denmark) and shaken. Aliquots of these suspensions were spread directly onto GVPC agar or were heated at 50°C for 30 minutes prior to being plated onto GVPC agar. Samples were incubated as described in Section 2.10.1a. Isolation and quantification of heterotrophic bacteria were established as described in Section 2.10.1b.

Additional biofilm samples were collected by swabbing a defined area and resuspending the collected material in 2 ml of sterile PAS. The suspensions were processed as above.

2.10.3 Bacterial identification

2.10.3a *Legionella*

The identity of *Legionella* isolates was confirmed by the direct fluorescent antibody

(DFA) test using anti-*L. pneumophila* serogroup 1 monoclonal antisera directly linked to FITC (reagents obtained from the Laboratory of Microbiological Reagents, Colindale, London). Antigen preparation was the same as described in section 2.1.6. The conjugated antibody (5 μl) was added, and the slide was incubated at 37°C for 1 hour in a moist atmosphere (in the absence of light). Subsequent to incubation, the slide was washed twice with PBS, once with distilled water (5 minutes for each wash), blotted and dried at 37°C for 10 minutes in the dark. The slide was mounted in fluorescence preserving glycerol/PBS mounting medium (Citifluor Ltd., London), and viewed by epi-illumination using ultraviolet light of 336 nm. *Legionella* isolates were also tested using a latex agglutination kit (Prolab, Bootle) containing antisera to *L. pneumophila* serogroups 1-14 and *L. micdadei.*

2.10.3b Heterotrophs

Heterotroph isolates were identified on the basis of colony morphology, Gram stain characteristics and by using the API-20NE (Biomerieux, France) and BIOLOG GN (Biolog, Hayward, California) bacterial identification systems.

2.10.4 Data analysis

Data was collected as described previously in Section 2.8.4. The comma delimited ASCII data files generated by the logging software were transferred into a statistics package for data management and analysis. Bacterial counts and water chemistry data were also input to the statistical analysis software. The statistics program used was Unistat 3.0 (Unistat Ltd., London). Logarithmic transformations were used in the statistical analysis to normalise count data. Product-moment (Pearson's) correlation coefficients were calculated. Legionella counts determined immediately after inoculation were omitted from the correlation analysis.

2.11 COOLING TOWER OPERATION

The cooling tower was operated under different regimes designed to mimic operational conditions likely to be encountered in the field.

2.11.1 Modes of operation:

2.11.1a Mode 1: Spring/Autumn cooling demand

The Spring/Autumn cooling demand was mimicked by keeping the heat exchanger at around 34°C with the recirculation pump only operating 2 hours per day. During this time the fan was run at low speed giving an air speed of approximately 0.5 $m.s^{-1}$. This mode of operation of the fan simulated natural convection, when a tower is operated with the pump running but with the fan off, a situation which occurs when there is a low cooling demand. The heat load was increased during the 2 hours of tower operation by increasing the water bath set point and switching on booster heating elements.

The experiments in which the tower was operated in Mode 1 were carried out prior to the modifications described in Section 2.8.3a. However, when this mode of operation was repeated with the new fan and ducting arrangement it was not possible to reliably achieve an air speed of 0.5 m s^{-1}. This was due to fluctuations of air pressure at the filters caused by outside weather conditions which in turn caused the pressure sensor relay to close the air intake damper. This had to be avoided to prevent damage to the fan and other parts of the system. It was, therefore, necessary to modify the operating protocol for the repeat Mode 1 experiments, and was referred to as Mode R1.

2.11.1b Mode R1: Spring/Autumn cooling demand

As in Mode 1 the heat exchanger was kept at around 34°C with the recirculation pump only operating 2 hours per day. During this time the fan was run to give an air speed of approximately 1 m s^{-1}. The heat load was increased during the 2 hours of tower

operation by increasing the water bath set point and switching on booster heating elements.

2.11.1c Mode 2: Continuous steady demand during the working day

The tower was operated continuously for around 8 hours per day. Heat load was applied throughout the period with booster heating elements turned on. The pump and fan were run continuously during this period, with the fan set at maximum (giving an air speed in the tower of around 2.5 m s^{-1}). Air flow was limited to prevent exceeding the face velocity of the HEPA filters.

2.11.1d Mode 3: *Intermittent demand*

The pump was run for 2 hours on, 2 hours off and 2 hours on; the air speed was approximately 1 m s^{-1} for the duration of the pump on-time with the exception of a 20 minute interval in the middle of this on-time when it was increased to around 2.5 m s^{-1}. Heat load was applied throughout the operation period to maintain a temperature at the heat exchanger of approximately 34°C during the periods that the tower was inactive, the waterbath set-point was increased and the booster heaters turned on during tower operation.

During each mode the fan was run for a short period after the pump was switched off to dry the tower and filters.

2.12 DISINFECTION TRIALS

The efficacy of the emergency disinfection protocol *i.e.* "... a level of 50 mgl^{-1} (mg l^{-1} ≡ parts per million [ppm]) of free available chlorine should be maintained for at least 4 hours", recommended in the report of the DoH Expert Advisory Committee on Biocides (Department of Health, 1989) was evaluated.

Volumes of sodium hypochlorite (Chloros, Bartlett and Sons Ltd., Bristol) were added to give a chlorine concentration in circulating water of 50 ppm. The pump was activated and the water circulated to allow adequate mixing of chlorinated water. A water sample was then drawn off and the level of free available chlorine in the sample measured using the Palin test (Lovibond, Salisbury, Wilts.). Further volumes of sodium hypochlorite were added, as required, to maintain the free chlorine content of the water at 50 ppm. After 4 hours of circulating the cooling water containing 50 ppm through the system (pump on, fan off), water samples were collected and processed for isolation of legionellae and HPC bacteria (as described in Section 2.10). No attempt was made to inactivate the chlorine prior to microbiological evaluation because the bacteria were rapidly filtered out of the sample and resuspended in chlorine free diluent. Samples were also taken by swabbing the packing and the sediment from the heat exchanger and pond. These were tested for the presence of legionellae and heterotrophic bacteria.

3.1 CONTINUOUS CULTURE PHYSIOLOGY EXPERIMENTS

Strains 74/81 and Corby grew well in ABCD broth which has been found suitable for the growth of *L. pneumophila* serogroup 1 (Knoxville 1), *L. bozemanii* and *L. dumoffii* in batch culture (Pine *et al.*, 1986). However growth of both strains was critically affected by dissolved oxygen tension (DOT). Preliminary chemostat experiments using several different levels of DOT indicated that maximal biomass concentration occurred at a DOT of 4% of air saturation at 30°C (equivalent to 0.31 mg O_2 l^{-1}). This is consistent with reports of oxygen toxicity in *L. pneumophila* (Locksely *et al.*, 1982; Pine *et al.*, 1986). Since the DOT is influenced by temperature the oxygen concentration was maintained at 0.31 mg l^{-1} during growth at 24°C and 37°C by adjusting the DOT to 3.6% and 4.5% of air saturation, respectively. To promote growth the medium was deoxygenated with nitrogen until the DOT decreased to 4% of air saturation, prior to inoculation. The DOT was kept low by restricting agitator speed until growth was established. Steady state growth, defined by a constant culture turbidity and cell yield (g dry wt l^{-1}), was achieved within 10 generations.

3.1.1 Nutrient utilisation

Analysis of the clarified culture supernatants of strain 74/81 showed that although serine was the principal source of carbon and energy, it remained in excess during steady state growth at all temperatures (Table 3.1), in agreement with the findings of George *et al.* (1980) for batch culture of *L. pneumophila* using a similar medium. Metabolism of approximately 18.5 mM serine was accompanied by the production of approximately 15 mM ammonia. Increasing the growth temperature from 30°C to 37°C did not cause any significant change in the metabolism of serine; ammonia production, however, did increase to 16.5 mM. Lowering the growth temperature to 24°C slightly decreased serine utilisation.

Only a few amino acids were completely depleted at 24°C but several more could not be detected in the culture supernatants at the higher growth temperatures.

Cystine was detected in culture filtrates and unused ABCD broth, although it was not present in the formulation of the medium. Its presence was attributed to auto-oxidation of cysteine catalysed by the iron in the medium (*c.f.* Section 3.3.1).

Similar results were obtained with the clinical strain Corby.

3.1.2 Growth yield

The results of representative chemostat runs using each strain are given in Table 3.2. Similar results were produced on repeat experiments using fresh inocula, three runs were carried out using strain 74/81 as the inoculum and two with strain Corby. The culture turbidity, biomass, molar growth yield for serine (Y_{serine}) and viable count indicated that 74/81 grew well at a temperature of 24°C (Table 3.2). This correlated with a high specific rate of serine metabolism (q_{serine}). Raising the temperature from 24°C to 30°C caused an increase in the culture turbidity, biomass, Y_{serine} and viable count, which was associated with a 25% decrease in q_{serine} as the growth efficiency improved. The $Y_{potassium}$ and $Y_{phosphate}$ also increased by 93% and 27% respectively. Growth of strain 74/81 appeared to be optimal at 30°C since increasing the temperature to 37°C resulted in a lower turbidity and poorer recovery of viable bacteria. However, the biomass, Y_{serine} and q_{serine} were less affected, suggesting that the mean weight of individual cells had increased. Corby exhibited a much greater increase in yield when grown at 37°C rather than at 24°C, as indicated by an increase in cell dry weight of 121% and an increase of 54% in Y_{serine} (Table 3.2).

Although the cultures were transiently brown pigmented in the initial batch culture phase, no pigmentation was observed during continuous culture. However, when continuous cultures were pulsed with excess tyrosine brown pigmentation was

Table 3.1 Concentration of nutrients (mM) in unused ABCD broth and in culture filtrate of *L. pneumophila* 74/81 grown in a chemostat at D = 0.08 h^{-1}, with dissolved oxygen concentration of 0.31 mgl^{-1} and pH 6.9, at the temperatures shown. Figures for unused broth are means of 11 samples and filtrate figures are means of three samples. Standard deviations were all less than 15% of means.

Nutrient	Unused medium	Culture Filtrate Growth Temperature (°C)		
		24	30	37
Iron[a]	0.16	0.16	0.16	0.16
Potassium	21.85	20.46	20.89	23.38
Zinc[a]	0.08	0.08	0.08	0.08
Ammonium	7.30	22.25	21.67	24.00
Phosphate	1.64	1.02	0.99	1.07
Alanine	0.93	1.16	0.60	0.60
Arginine	0.54	ND[b]	ND	ND
Asparagine	0.66	ND	ND	ND
Aspartate	0.62	0.18	0.25	0.08
Cysteine	0.68	0.26	0.32	0.58
Cystine	0.12	0.09	0.02	0.22
Glutamate	0.59	0.10	ND	0.13
Glutamine	0.54	0.10	ND	ND
Glycine	0.97	0.88	0.74	0.86
Histidine	0.36	0.06	0.05	0.05
Isoleucine	0.70	0.06	ND	0.05
Leucine	0.66	0.02	ND	0.04
Lysine	0.44	0.06	ND	ND
Methionine	0.59	0.20	0.06	0.04
Phenylalanine	0.41	ND	ND	ND
Proline	0.82	ND	ND	ND
Serine	19.03	0.57	0.24	0.21
Threonine	0.63	0.04	ND	ND
Tryptophan	0.28	0.20	0.22	0.22
Tyrosine	0.12	ND	ND	ND
Valine	0.76	0.12	ND	0.08

[a] Differences in the concentrations of iron and zinc between the medium and culture filtrate are most probably below the detection level of the procedure used.

[b] ND = not detected.

Table 3.2. Growth characteristics of two different strains of *Legionella pneumophila* grown in continuous culture at $D=0.08h^{-1}$, 0.31mg dissolved oxygen l^{-1} and pH 6.9, at various temperatures.

	Growth Temperature (°C)				
	24		30	37	
	74/81	Corby	74/81	74/81	Corby
OD_{540}	2.7	1.8	3.6	2.8	3.4
Biomass [g (dry wt.) l^{-1}][a]	0.946	0.595	1.262	1.054	1.317
CFU ml^{-1} ($x10^9$)[b]	3.8 ± 0.62	1.5 ± 0.39	7.2 ± 0.85	2.5 ± 0.50	6.2 ±0.79
Y_{serine} (g mol^{-1})	51.2	45.7	67.2	56.0	70.2
q_{serine} (mmol g^{-1} h^{-1})	1.6	1.8	1.2	1.4	1.2
PHB (% of cell dry wt.)	4.3	ND	2.9	1.5	ND

ND, not determined.

[a] of triplicate samples, standard deviations less than 5% of mean in all cases except Corby grown at 24°C, where s was 9.7% of mean.

[b] ± standard error of the mean, SEM.

observed, probably due to melanin formation following tyrosinase activity (Baine *et al.*, 1978).

3.1.3 Growth limiting nutrient

Several amino acids were completely depleted at all culture temperatures, these were arginine, asparagine, proline, phenylalanine, proline and tyrosine, with threonine only being detectable at a low level in 24°C culture filtrate. Similar results were obtained for strain Corby. To ascertain whether any of these were actually growth limiting, the concentrations of all the amino acids except serine were doubled in the incoming medium. The biomass concentration increased immediately and the oxygen concentration decreased to zero, becoming growth limiting, suggesting that growth in the original ABCD medium was limited by one of the amino acids but not serine. Supplementing the medium with a cocktail of the amino acids which were completely depleted at all three temperatures (arginine, asparagine, proline, phenylalanine, tyrosine and threonine; threonine was detectable at 24°C , but at a very low concentration), similarly increased the yield and also resulted in oxygen limited growth, indicating that one of these was growth limiting. When MOD1 was substituted for ABCD as the culture medium at 37°C the turbidity of the culture dropped to 1.8 at 540 nm and the cell dry weight was reduced to 0.758 g l^{-1}. The turbidity and dry weight of the culture was monitored before and after the addition of supplements of the above amino acids to determine if there was any increase in biomass concentration. No increase in biomass concentration was noted with any of the amino acids tested except for tyrosine. When tyrosine was added to the chemostat the OD_{540} increased to 2.2 and the dry weight increased to 0.894 g l^{-1}, an increase of around 18%. The addition of tyrosine to the culture vessel resulted in the formation of a brown pigment, the absorbance maximum of this was 295 nm which is similar to that of tyrosine (293.5 nm, in NaOH) and melanin (301 nm, in KOH), but it did not absorb significantly at 540 nm and so did not account for the rise in the turbidity of the culture. To confirm the role of tyrosine as the growth limiting substrate ABCD broth was supplemented with tyrosine. Doubling the

concentration of tyrosine in ABCD broth (to 0.56 mM) gave an increase in OD_{540} of 0.4 and an increase in dry weight of approximately 11%, confirming tyrosine as the growth limiting nutrient. These findings would also, suggest that the other amino acids in the cocktail are not essential. Further supplementation of the medium with serine resulted in an additional increase in biomass concentration, indicating that serine was then limiting. Similar results were obtained at each growth temperature.

The essential trace elements, including iron and zinc, remained in excess throughout the experiments.

3.1.4 Morphology

Changes in the growth temperature resulted in morphological differences (Figure 3.1). At 24°C short fine rods, approximately 1.3 μm x 0.25 μm were observed, many of them bearing a single sub-polar flagellum (3.6 - 5 μm long). Free flagella were also present, these having presumably been sheared off due to the rapid agitation of the culture. No flagella were observed at either 30°C or 37°C, either attached to the cells or free in the culture. Some of the cells appeared slightly elongated at 30°C (1.8 μm x 0.4 μm) and intracellular inclusions could be seen in the majority. The culture exhibited a high degree of pleomorphism at 37°C with long rods predominating. These were up to 20 μm in length and intracellular inclusions of up to 0.5 μm diameter were observed in a proportion of the cells.

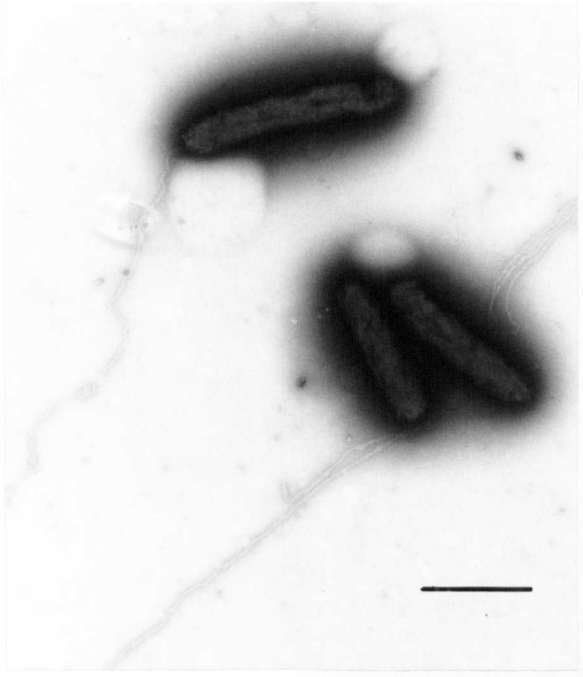
The negatively stained appearance of inclusion granules in electron micrographs, either remaining inside or released from the cell envelope of lysed bacteria suggested that they were indeed solid granules and not vacuoles (Figure 3.2). Discrete granules were also observed with a light microscope after staining the cells with the lipophilic stain Sudan Black and as refractile inclusion bodies when observed by DIC microscopy. Analysis of chloroform extracts of lyophilised bacteria by gas chromatography mass spectrometry (GC-MS) and by ^{13}C nuclear magnetic resonance (NMR) spectroscopy

confirmed the presence of the well known microbial carbon storage compound, polyhydroxybutyrate (PHB). The isobutane chemical ionisation spectrum of the methyl ester of the extract indicated the molecular weight, with the protonated molecular ion at m/z 119 dehydration of which produced a prominent fragment at m/z 101, and elimination of the methoxy group generated m/z 87 which are consistent with the methyl ester of ß-hydroxybutyrate (Figure 3.3). The electron impact mass spectrum also demonstrated significant cleavage ions which were diagnostic of methyl ß-hydroxybutyrate. In addition the GC retention time, electron impact spectrum and the chemical ionisation spectrum of the extract from *L. pneumophila* were identical to those produced following methanolysis of authentic PHB from *Alcaligenes* sp. Moreover, the proton decoupled ^{13}C NMR spectrum of the material extracted from *L. pneumophila* was also identical to that of authentic PHB from *Alcaligenes* sp. (Figure 3.4). This polymer comprised 4.3% of the dry weight of cells grown at 24°C but decreased to 1.5% at 37°C (Table 3.2). The proportion of cells containing granules did not, however, correlate with the PHB content of the biomass, therefore other possibilities were investigated. Thin sections of bacteria were examined by electron microscopy after treatment with a polysaccharide-specific silver proteinate stain (Thiéry, 1967). The intracellular granules did not take up the stain which indicates that they are not composed of glycogen or other polysaccharides.

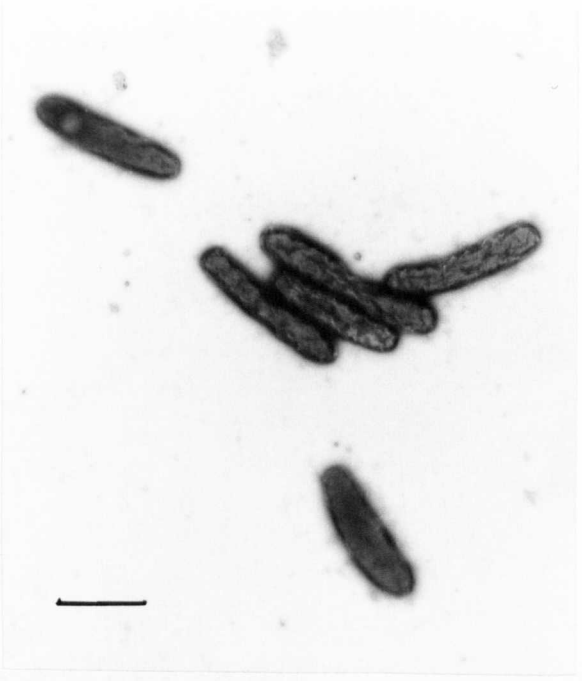
Extracellular material is frequently observed in Gram-negative bacteria including *L. pneumophila* grown in complex media (Dennis, 1986), but none was observed in the present study.

Figure 3.1 Transmission electron micrographs of *L. pneumophila* 74/81 grown under defined conditions in continuous culture at (a) 24°C, showing short rods with a single polar flagellum (F); (b) at 30°C, cells of similar size and shape, but without flagella; and (c) at 37°C, the culture exhibiting pleomorphism, with the presence of both long and short rods. Bar marker, 1 μm.

(a)



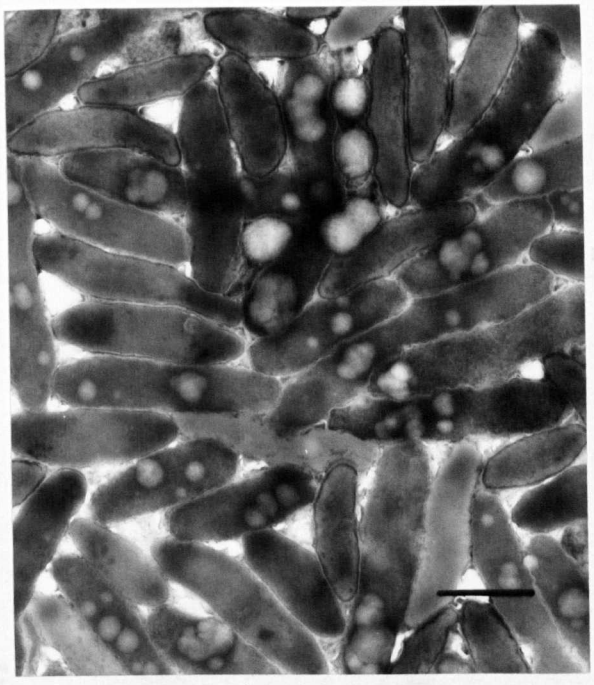
(b)



(c)

Figure 3.2 Transmission electron micrographs of *L. pneumophila* 74/81 grown under defined conditions in continuous culture at various temperatures, showing the presence of intracellular granules. (a) A field showing cells grown at 30°C, most cells have at least one granule, and (b) 37°C, showing the cell envelope of long cell containing several granules. Bar marker, 1 μm.

(a)



(b)

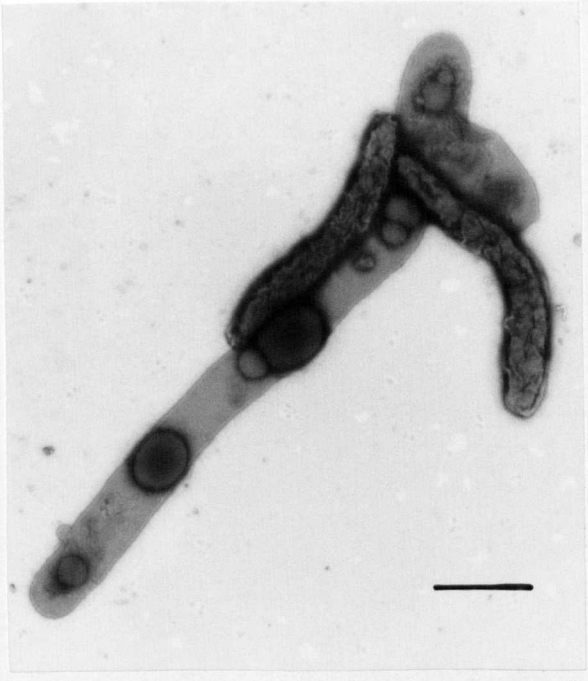


Figure 3.3 (a) Electron impact and (b) isobutane chemical ionisation spectra of the methanolysate of the extract from *L. pneumophila*.

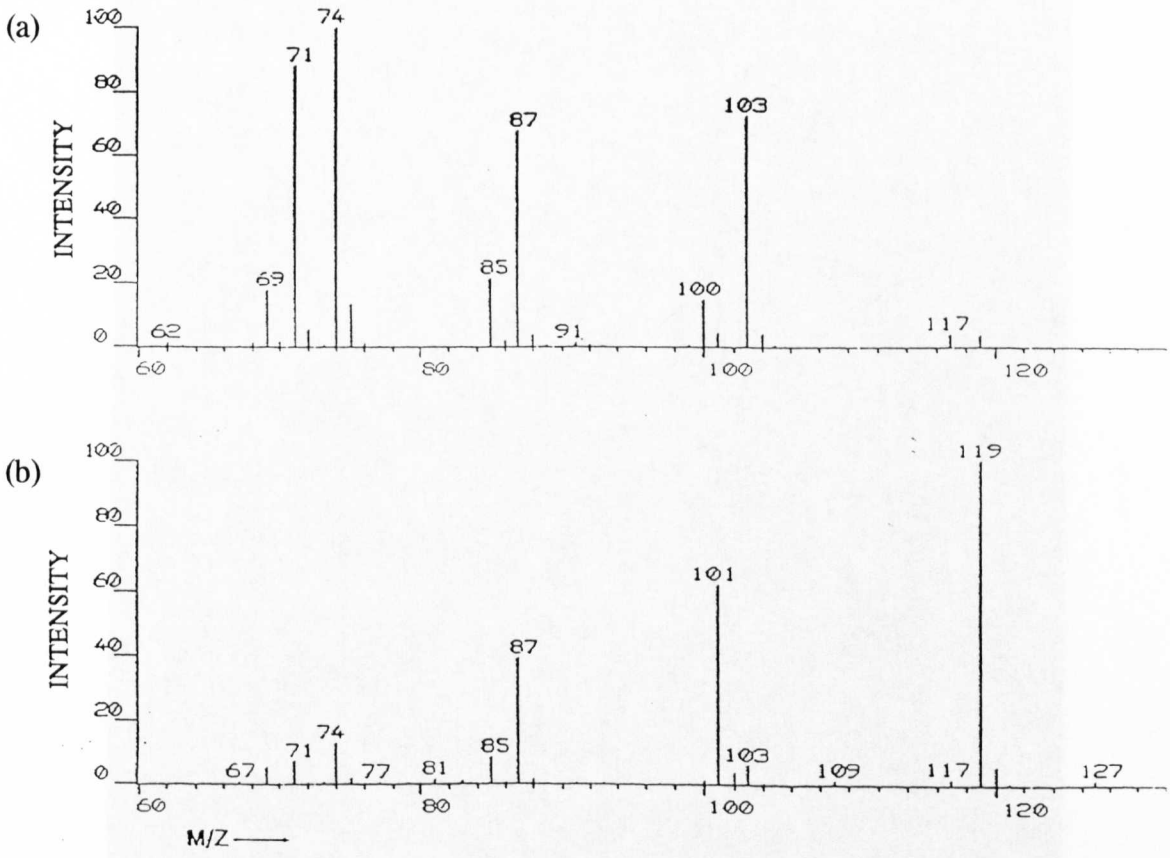
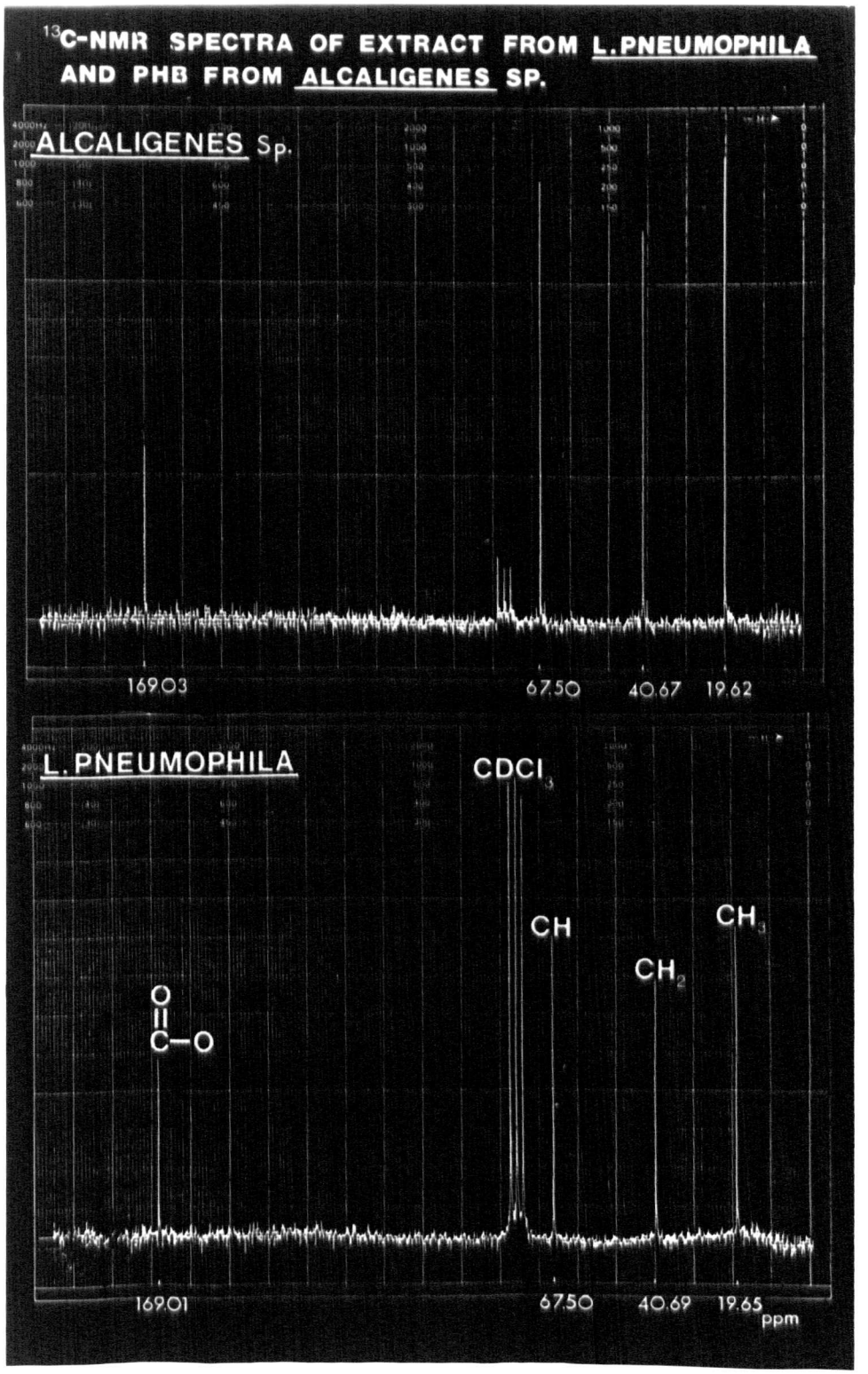


Figure 3.4 ^{13}C NMR spectra of extract from *L. pneumophila* and PHB from *Alcaligenes* sp.



3.1.5 Membrane fatty acid content

The fatty acid composition of *L. pneumophila* is unusual among Gram negative bacteria because of the predominance of branched chain acids, of which 14-methyl pentadecanoic acid (i-16:0) was the most abundant when the organism was grown on BCYE agar at 37°C (Moss *et al.*, 1977). A similar fatty acid profile was obtained in continuous culture at 37°C using defined medium (Table 3.3). However, as the temperature of the culture was lowered, there was a progressive reduction in the relative proportion of saturated acids, particularly i-16:0 and i-14:0, and a corresponding increase in unsaturated acids so that at 24°C the most abundant acid was 9-hexadecenoic acid, and the proportion of i-16:0 had fallen to less than 20% of the total (Table 3.3). The 9,10 methylenehexadecanoic acid (cyclopropane 17), a significant constituent of legionellae grown on BCYE agar (Moss *et al.*, 1977) was barely detectable in bacteria grown at 37°C in continuous culture and was absent at 24°C.

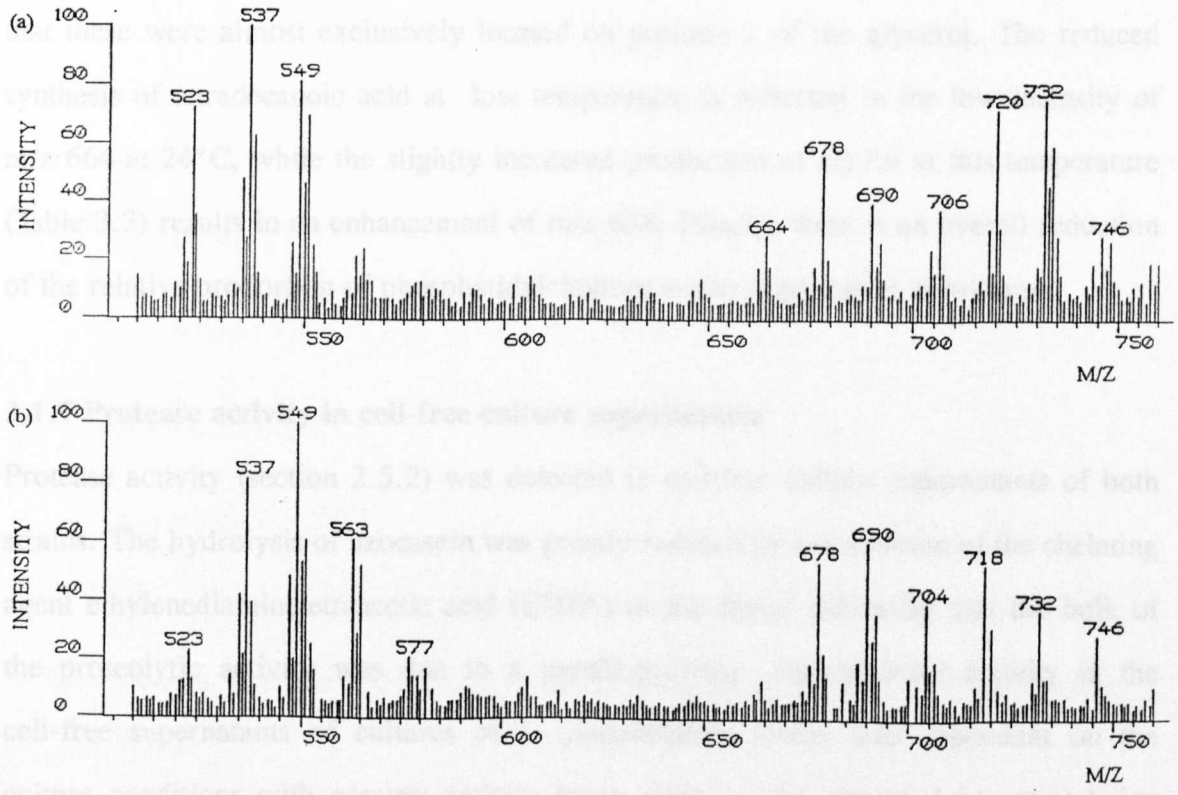
3.1.6 Phospholipid analysis

Fast atom bombardment mass spectrometry was used to record profiles of intact phospholipids at the various temperatures. Two representative spectra are shown in Figure 3.5. Signals in the region m/z 664-706 represent the protonated molecular ions of phosphatidylethanolamines and N-methylphosphatidylethanolamines, while those between m/z 718 and 748 correspond to phosphatidylcholines, all varying in their pattern of fatty acid substitution. The cluster of ions between 523 and 563 are fragments derived from the loss of the polar head group from phosphatidylethanolamines and phosphatidylglycerols, with charge retention on the glycerol backbone fragment.

Table 3.3 Influence of temperature on the membrane fatty acid composition (%) of *L. pneumophila* grown in continuous culture at D = 0.08 h^{-1}, 0.31 (mg dissolved oxygen) l^{-1} and pH 6.9.

	Fatty acid									
Temperature (°C)	i14:0	a15:0	i16:1	i16:0	16:1	16:0	a17:1	a17:0	17:0	18:0
37	2	9	1	31	23	11	<1	11	6	8
30	1	7	2	32	30	10	1	6	2	6
24	<1	7	4	17	30	10	3	15	4	4

Figure 3.5 Part of the positive ion FAB mass spectra of *L. pneumophila* strain 74/81 grown under defined conditions in continuous culture at (a) 37°C and (b) 24°C.



Several differences are apparent between the 37°C and the 24°C samples. Firstly, as the temperature is reduced the proportion of phospholipids containing an unsaturated fatty acid substituent is increased; thus the signals m/z 690, 704 and 718 are significantly enhanced at low temperature. This tendency is particularly pronounced in the phosphatidylcholines as the signals m/z 734 and 748 are virtually absent at 24°C. In general the unsaturated phospholipid species included only one double bond-containing substituent. Digestion with phospholipase A_2, followed by FAB-MS demonstrated that these were almost exclusively located on position 2 of the glycerol. The reduced synthesis of tetradecanoic acid at low temperature is reflected in the low intensity of m/z 664 at 24°C, while the slightly increased production of ai17:0 at this temperature (Table 3.3) results in an enhancement of m/z 678. Finally, there is an overall reduction of the relative proportion of phosphatidylcholines as the temperature is lowered.

3.1.7 Protease activity in cell-free culture supernatants

Protease activity (section 2.5.2) was detected in cell-free culture supernatants of both strains. The hydrolysis of azocasein was greatly reduced by the addition of the chelating agent ethylenediaminetetraacetic acid (EDTA) to the digest indicating that the bulk of the proteolytic activity was due to a metalloprotease. Azocaseinase activity in the cell-free supernatants of cultures of *L. pneumophila* Corby was dependent on the culture conditions with greatest activity (even allowing for increased biomass) being present in cultures grown at 37°C under iron-replete conditions (Table 3.4). The protease activity in supernatants of cultures grown at 24°C was 146% of that in cultures grown at 37°C when related to total protein in the supernatant, however when related to the biomass of the culture the activity in the culture at 24°C was approximately 65% that of culture grown at 37°C suggesting that azocasein hydrolysing enzyme makes up a larger proportion of the extracellular protein at 24°C. A similar situation occurred in iron-limited cultures subsequently supplemented with 2.5 μM iron with activity related to biomass being approximately 44% of the value for the culture iron-replete at 37°C, but when activity was related to the total protein in the supernatant the value obtained

Table 3.4 Azocaseinase activity in cell free culture supernatants of *L. pneumophila* Corby grown in a chemostat at D = 0.08 h^{-1}, 0.31 (mg dissolved oxygen) l^{-1} and pH 6.9.

Culture conditions	Azocaseinase activity, Units[a] (U) related to:	
	Biomass[b] U $mg_{(dry\ weight)}^{-1}$	Extracellular protein[c] U $\mu g_{(protein)}^{-1}$
Iron-replete, 24°C	168.6	22.2
Iron-replete, 37°C	258.9	15.2
Iron-limited, 37°C	65.5	3.7
Iron-limited[d], 37°C	114.6	23.2

[a] One unit of protease activity is the amount of enzyme which results in an increase in A_{440} of 0.001.

[b] Protease activity in supernatant divided by the biomass concentration of the culture.

[c] Protease activity in supernatant divided by the total protein concentration in the supernatant.

[d] Culture was growing in MOD2 broth which was then supplemented with 2.5 μM iron and biomass concentration increased to a level similar to that achieved when growing in ABCD broth. Samples were taken when new steady-state had been achieved.

was around 153% that of the culture grown iron-replete without a previous period of iron-restriction. When the culture was grown iron-limited at 37°C the azocaseinase activity was approximately 25% and 24%, related to biomass and total extracellular protein respectively, of the activity in the supernatant of the culture grown at 37°C iron-replete.

3.2 THE INFLUENCE OF GROWTH TEMPERATURE ON MEDIAN LETHAL DOSE (LD_{50})

The preliminary LD_{50} values obtained for both strains grown at 24°C and 37°C are shown in Table 3.5. These results indicated that both strains were more virulent when grown at 37°C rather than 24°C. The virulence of strain 74/81 was greatly reduced when the growth temperature was lowered to 24°C (Table 3.5): at 37° the LD_{50} was $\log_{10}$ 4.3 CFU, however when the guinea pigs were challenged with similar doses of cells grown at 24°C none of the animals died. On returning the culture temperature to 37°C the virulence returned to a level similar to that measured before the drop in temperature, giving a LD_{50} of $\log_{10}$ 4.8 CFU. A similar trend was obtained during repeat experiments using fresh inocula of 74/81 (data not shown). When the experiment was repeated using a culture of Corby similar results were obtained (Table 3.5), *i.e.* a reversible reduction of virulence on shifting the temperature of the culture from 37°C to 24°C.

Since the preliminary experiments had indicated that growth temperature could reversibly modulate the virulence of two strains of *L. pneumophila* and that this phenomenon was reproducible, it was considered necessary to test the validity of these observations by undertaking a more rigorous experiment in which a higher degree of statistical confidence could be assigned to the results obtained. This was done with a culture of *L. pneumophila* Corby. When grown at 37°C the culture was shown to be significantly ($P < 0.05$) more virulent than the same culture grown at 24°C, with all

Table 3.5 Approximate LD_{50} values for two different strains of *L. pneumophila* grown in chemostat culture at different temperatures. D = 0.08 h^{-1}, pH 6.9 and dissolved oxygen concentration 0.31 mg oxygen l^{-1}.

Temperature (°C)	LD_{50} (Log_{10} CFU)	
	Strain:	
	74/81	Corby
37	4.3	4.0
24	> 6.0[a]	> 5.4[a]
37[b]	4.8	4.3

[a]Number of bacteria inhaled and retained at highest concentration did not cause death.

[b]Culture returned to 37°C after being at 24°C.

other culture conditions remaining constant (Table 3.6). Moreover, when the temperature was returned to 37°C there was a statistically significant ($P < 0.05$) increase in virulence, with a LD_{50} value obtained which was similar to the one noted at the start of the experiment (Table 3.6). It should be noted that the LD_{50} values obtained for cultures grown at 37°C which had previously been cultured at 24°C were consistently slightly higher than for cultures which had not undergone low temperature growth, and in the second experiment this increase was statistically significantly different ($P < 0.05$) (Table 3.6). However the magnitudes of these differences were small and the confidence intervals for the LD_{50} values in the second experiment just failed to overlap so this apparent difference may be attributable to inconsistencies which are inevitable in such estimations of virulence.

3.3 IRON LIMITATION

3.3.1 Iron-limited metabolism

Steady-state growth was obtained under conditions of iron restriction when the medium supplied to a culture of *L. pneumophila* Corby growing in a chemostat was changed from ABCD broth to its low iron derivative MOD2 broth. As expected the biomass concentration decreased, as indicated by a drop in turbidity of the steady-state culture from 3.4 at 540 nm to 1.4 with a drop in cell dry weight from 1.320 g l^{-1} to 0.583 g l^{-1}. (a decrease of 56%) MOD2 broth contained trace amounts of iron (as contaminants of other medium components): the concentration of iron in this batch of MOD2 was determined to be 0.3 μM by electrothermal ioniser atomic absorption spectrometry. When sterile ferrous sulphate solution was titrated into the medium reservoir it was found that addition of 2.5 μM of iron was sufficient to result in an increase in OD_{540} to 3.4, and a dry weight of 1.372 g l^{-1}, similar values as when the culture was grown in ABCD broth. This agreed with the value obtained for iron utilised by cultures grown in ABCD broth (mean value of approximately 3 μM). Further additions of ferrous sulphate to give iron concentrations of up to 25 μM did not result in any further

Table 3.6 LD_{50} values and their 95% confidence intervals (where appropriate) obtained for *L. pneumophila* Corby grown in chemostat culture at 24°C and 37°C. D = 0.08 h^{-1}, pH 6.9 and dissolved oxygen concentration 0.31 mg oxygen l^{-1}.

Temperature (°C)	LD_{50} (Log_{10} CFU)	95% Confidence Interval of LD_{50}[a] (Log_{10} CFU)
37	3.30	2.98 to 3.82
24	> 5.60[b]	NA[c]
37[d]	4.25	4.02 to 4.52

[a] 95% fiducial limits for the median lethal dose (LD_{50}) calculated by the method of Finney (1964).

[b] Number of bacteria inhaled and retained at highest dose did not cause death.

[c] NA, not appropriate.

[d] Culture returned to 37°C after being at 24°C.

increase in turbidity, so it was concluded that 2.8 μM iron was sufficient to support maximal biomass production with some other nutrient being growth limiting. Under iron-limitation, Y_{iron} increased approximately 4-fold, from 0.47 x 10^{-6} g mol^{-1} to 1.94 x 10^{-6} g mol^{-1} which suggests increased efficiency of iron metabolism. However Y_{serine} was reduced by 50% to 35.1 g mol^{-1}, indicating a reduction in the efficiency of serine metabolism. The $Y_{phosphate}$ value doubled under iron-restriction (1.55 to 3.07 g mol^{-1}). The total viable count of *L. pneumophila* under iron-limited conditions was 1.77 x 10^9 CFU ml^{-1}, which was a 72% reduction compared with iron-replete counts.

All amino acids were present in excess, with the exceptions of asparagine, glutamine and proline, in filtrates of iron-limited cultures. Cystine was not detected in unused MOD2 nor in filtrates, this was probably due to the much reduced concentration of iron present in the medium available to catalyse the auto-oxidation of cysteine.

3.3.2 Iron-limited morphology

Under iron excess conditions (*i.e.* growth on ABCD broth) the culture had a slight brown pigmentation, this was not present when cultures were grown in MOD2 broth, even after supplementation with iron to relieve iron-restriction. Iron-limited cultures consisted of short rods (1 to 3 μm) which was in contrast to iron-replete cultures grown at 37°C which exhibited pleomorphism with a large proportion of elongated cells (see section 3.1.3). After iron-restriction was lifted the cultures regained a similar degree of pleomorphism as with cultures grown in ABCD broth. Colony morphology and reaction with *L. pneumophila* serogroup 1 antiserum were similar for iron-limited and iron-replete cultures. Suggesting that the reactive epitope is conserved in either environment. Similar to iron-replete, 37°C cultures, iron-limited cultures at this temperature were also unflagellated.

3.3.3 Siderophore assay

Siderophore activity was detected in cultures grown under iron-limitation but not in cultures grown in conditions of iron excess. The siderophore activity in the supernatants of cultures grown under iron-limitation was equivalent to that of 10.32 μM of desferoxamine methanesulphonate (Table 3.7). The rate of iron transfer from CAS to the siderophore is dependent on the structure of the siderophore (Schwyn & Neilands, 1987). Equilibrium was attained in the assay mixtures of iron-limited *L. pneumophila* culture supernatants after approximately 140 minutes suggesting that the siderophore present was a polyamino carboxylic acid.

3.3.4 Effect of iron-limited growth on virulence

When cultures of *L. pneumophila* Corby grown under conditions of iron-limitation were used to provide an aerosol challenge for guinea pigs as described in section 2.1.10 there was no subsequent mortality. This loss of virulence was reversed by the subsequent addition of iron to the medium reservoir (Table 3.8). These results were reproducible in a repeat experiment using a fresh inoculum of *L. pneumophila* Corby. Subsequently, more extensive experiments (undertaken with a colleague) established the statistical validity of this observation (James *et al.*, 1995).

Table 3.7 Siderophore activity in cell free culture supernatants of *L. pneumophila* Corby determined using the universal assay of Schwyn and Neilands and expressed as an equivalent to the activity of desferoxamine methanesulphonate (DFOM).

Culture conditions	Siderophore concentration [a] (μM)
Iron-replete grown at:	
24°C	0
37°C	0
Iron-limited grown at:	
37°C	10.32[b]

[a] Siderophore activity expressed as that equivalent to desferoxamine methanesulphonate.

[b] Mean of three samples, standard deviation was 7.4% of the mean.

Table 3.8 Approximate LD_{50} values for *L. pneumophila* Corby grown in chemostat culture under conditions of different iron availability at 37°C. D = 0.08 h^{-1}, 0.31 mg dissolved oxygen l^{-1} and pH 6.9.

Culture conditions	LD_{50} (Log_{10} CFU)
Iron-replete,	4.0
Iron-limited,	> 4.7[a]
Iron-replete[b],	3.8

[a] Highest dose retained was insufficient to cause mortality.

[b] Culture was growing in MOD2 broth was then supplemented with 2.5 μM iron; biomass concentration increased to a level similar to that achieved when growing in ABCD broth. Samples were taken when a new steady-state had been achieved.

3.4.1 Genus metabolism

When the multiwell plates were incubated aerobically some of the colour reactions were not visible after 24 hours, only becoming apparent after longer incubation. For example, incubating *L. pneumophila* serogroup 1 NCTC 11192 at the manufacturer's recommended concentration in the multiwell plates took 72 hours in air to give 15 positive reactions (Table 3.9). This number is considered by the manufacturer to be rather low for an accurate resolution of species; also 72 hours incubation is considered by the manufacturer to be unacceptably long as the multiwell plates are not packaged aseptically thus spurious positive results may occur on prolonged incubation due to the presence of contaminants. Only ß-hydroxybutyrate and methylpyruvate were recorded as giving a positive reaction within 24 hours for the various *L. pneumophila* serogroups (data not shown). In all subsequent experiments these substrates were consistently the most rapidly metabolised, as indicated by the formation of a deeper colour reaction than that produced from any of the other compounds in the panel. After 72 hours incubation more positive results were observed either by eye or using the microplate reader (Table 3.9). A possible explanation for this low level of metabolic activity is that *Legionella* species. are sensitive to low levels of hydrogen peroxide and superoxide radicals (Locksely *et al.*, 1982; Hoffman *et al.*, 1983). As stated earlier, *L. pneumophila* appears to be microaerophilic in laboratory media since it grows best in the continuous culture system described above when the dissolved oxygen tension was strictly controlled to 4% of air saturation. Consequently, resuspended legionellae were incubated in the BIOLOG system in a low oxygen environment by degassing anaerobic jars and regassing with 0.2 bar air, followed by 0.8 bar 5% (v/v) carbon dioxide in nitrogen. This gave a final oxygen concentration of approximately 4% (v/v) oxygen. Multiwell plates incubated in this environment gave definite reactions within 24 hours. Under these conditions seven additional substrates were metabolised: Tween 40 (polyoxyethylenesorbitan monopalmitate), Tween 80 (polyoxyethylenesorbitan

monooleate), monomethyl succinate, acetate, L-aspartate, glycyl-L-aspartate and urocanate. Two further substrates, succinate and gamma-aminobutyrate, gave variable reactions in replicate samples. L-alanylglycine was not metabolised by the *L. pneumophila* NCTC 11192 strain under microaerophilic conditions (Table 3.9).

Representatives of other *L. pneumophila* serogroups and a range of type strains of other *Legionella* species were investigated using this modified BIOLOG procedure. All of the *Legionella pneumophila* strains investigated were similarly affected by the concentration of oxygen, yielding the largest number of positive reactions in a 4% (v/v) oxygen environment. The serogroup 7 strain was the least reactive of the *L. pneumophila* strains tested (Table 3.9a). Nevertheless, even this strain metabolised 15 substrates, which gave sufficient resolution, in this study, to differentiate it to species level. All of the *L. pneumophila* strains consistently metabolised Tween 40, Tween 80, methylpyruvate, ß-hydroxybutyrate, α-ketobutyrate, α-ketovalerate, alaninamide, L-alanine, L-glutamate, glycyl-L-glutamate, L-leucine, L-proline, L-serine, L-threonine and urocanate (Table 3.9a). Variability between the profiles obtained for different *L. pneumophila* strains was noted (Table 3.9a), emphasising the need to incorporate profiles for as many stains as possible to achieve a stable and reliable database.

The ability to utilise Tween 40 and Tween 80 suggests that *L. pneumophila* possesses esterase activity similar to that reported by Muller (1981) and Nolte *et al.* (1982) except that the data presented here suggest that the strains investigated in this study are able to split esters of higher fatty acids, as indicated by the metabolism of Tween 40 and Tween 80. Interestingly, although urocanate is an intermediate in histidine metabolism, only one of the strains tested, the serogroup 5 strain (NCTC 11405), was able to metabolise histidine within the 24 hour incubation period (Table 3.9a). The strains NCTC 12000 and NCTC 11984, which belong to serogroups 10 and 13 respectively, took 48 hours in 4% (v/v) oxygen to metabolise the histidine.

Table 3.9 Substrate metabolism profiles of *L. pneumophila* serogroup 1 (NCTC 11192) incubated at 37°C in an atmosphere of either 4% (v/v) or 21% (v/v) oxygen with Page's amoebal saline as diluent.

	Incubation atmosphere			
	21% Oxygen			4% Oxygen
	24 hours[a]	72 hours	24 hours 2 x [biomass][b]	24 hours
Substrate				
Tween 40			+[c]	+
Tween 80			+	+
Methylpyruvate	+	+	+	+
Monomethyl succinate			+	+
Acetate			+	+
ß-hydroxybutyrate	+	+	+	+
α-ketobutyrate		+	+	+
α-ketovalerate		+	+	+
Succinate		+	+	V
Alaninamide		+	+	+
L-alanine		+	+	+
L-alanylglycine		+	+	
L-asparagine		+	+	+
L-aspartate			+	+
L-glutamate		+	+	+
Glycyl-L-aspartate			+	+
Glycyl-L-glutamate		+	+	+
L-leucine		+	+	+
L-ornithine			+	
L-proline		+	+	+
L-serine		+	+	+
L-threonine		+	+	+
Gamma-aminobutyrate			+	V
Urocanate			+	+

[a] Duration of incubation.

[b] BIOLOG plate inoculated with twice the biomass concentration.

[c] + and V denote positive or variable reactions, respectively, in replicate samples.

Table 3.9a Substrate metabolism profiles of *L. pneumophila* serogroups incubated at 4% (v/v) oxygen, 37°C for 24 hours, using Page's amoebal saline as diluent.

	Profiles of *L. pneumophila* strain representing serogroup[a]											
	1	2	3	5	6	7	8	10	11	12	13	14
Substrate												
Tween 40	+[b]	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+
Methylpyruvate	+	+	+	+	+	+	+	+	+	+	+	+
Monomethyl succinate	+	+	+									
Acetate	+		+					+			+	+
ß-hydroxybutyrate	+	+	+	+	+	+	+	+	+	+	+	+
α-ketobutyrate	+	+	+	+	+	+	+	+	+	+	+	+
α-ketovalerate	+	+	+	+	+	+	+	+	+	+	+	+
Propionate			+				+				+	+
Succinate	V	+	+	+	+		+					
Alaninamide	+	+	+	+	+	+	+	+	+	+	+	+
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+
L-alanylglycine		+	+	+	+		+	+	+		+	+
L-asparagine	+	+	+		+		+	+	+	+	+	+
L-aspartate	+		+									
L-glutamate	+	+	+	+	+	+	+	+	+	+	+	+
Glycyl-L-aspartate	+											
Glycyl-L-glutamate	+	+	+	+	+	+	+	+	+	+	+	+
L-histidine				+								
L-leucine	+	+	+	+	+	+	+	+	+	+	+	+
L-ornithine			+									
L-proline	+	+	+	+	+	+	+	+	+	+	+	+
L-serine	+	+	+	+	+	+	+	+	+	+	+	+
L-threonine	+	+	+	+	+	+	+	+	+	+	+	+
Gamma-aminobutyrate	V	+										
Urocanate	+	+	+	+	+	+	+	+	+	+	+	+

[a]*Legionella pneumophila* serogroup 1 (NCTC 11192), 2 (NCTC 11230), 3 (NCTC 11232), 5 (NCTC 11405), 6 (NCTC 11287), 7 (NCTC 11984), 8 (NCTC 11985), 10 (NCTC 12000), 11 (NCTC 12179), 12 (NCTC 12180), 13 (NCTC 12181) and 14 (NCTC 12174) are denoted by serogroups 1-14 (excluding 4 and 9) above.

[b]+ and V denote positive or variable reactions, respectively, in replicate samples.

Similar metabolic profiles were noted for the other *Legionella* species examined except that they generally gave fewer positive reactions than *L. pneumophila* strains. The most metabolically versatile isolate was the type strain of *L. longbeachae* which gave 18 positive reactions (Table 3.10). By contrast, *L. hackeliae* gave only 7 positive reactions. Of the 95 substrates in the BIOLOG panel, 26 different substrates were metabolised by one or more *Legionella* species, with all the strains tested metabolising methylpyruvate, ß-hydroxybutyrate and L-proline. The majority of the species incubated in 4% (v/v) oxygen metabolised the following, within 24 hours: Tween 40 (except *L. rubrilucens*), α-ketobutyrate (except *L. israelensis*), α-ketovalerate (except *L. hackeliae* and *L. micdadei*), alaninamide (except *L. hackeliae*), L-asparagine (except *L. hackeliae* and *L. micdadei*), L-glutamate (except *L. micdadei*), L-serine (except *L. dumoffii* and *L. hackeliae*) and L-threonine (except *L. dumoffii* and *L. rubrilucens*). Unlike the *L. pneumophila* strains none of the species metabolised acetate, propionate, aspartate, glycyl-L-aspartate, histidine, L-ornithine or gamma-aminobutyrate. Only *L. feeleii* metabolised glycyl-L-glutamate, *L. longbeachae* metabolised succinate, *L. bozemanii* and *L. longbeachae* metabolised L-leucine, and *L. bozemanii*, *L. israelensis* and *L. longbeachae* metabolised monomethyl succinate. Although the BIOLOG panel of substrates contains a number of sugars *e.g.* glucose, fructose, galactose and maltose, none of the strains tested reacted positively with any of these. This is not unexpected since previous reports indicate that legionellae have low levels of glucose uptake and catabolism (Tesh *et al.*, 1983; Keen & Hoffman, 1984). Although Weiss *et al.* (1980) reported that the Philadelphia 2 strain of *L. pneumophila* utilised glucose-1-phosphate, none of the strains in this study showed any indication of utilising either glucose-1-phosphate nor glucose-6-phosphate, however this may be due to insufficient reducing power being generated to cause detectable reduction of the tetrazolium dye.

3.4.2 Specificity and sensitivity

The metabolic profiles of all of the strains which had been incubated in 4% (v/v) oxygen for 24 hours were compared with the manufacturer's database and then entered

Table 3.10 Substrate metabolism profiles of selected *Legionella* species incubated in 4% (v/v) oxygen, at 37°C for 24 hours, using Page's amoebal saline as diluent.

	Profiles of *Legionella* species								
Substrate	boz[a]	dum	fee	hac	isr	lon	mic	pne	rub
Tween 40	+[b]	+	+	+	V	+	+	+	
Tween 80	+	+				+	+	+	
Methylpyruvate	+	+	+	+	+	+	+	+	+
Monomethyl succinate	+				+	+		V	
Acetate								V	
ß-hydroxybutyrate	+	+	+	+	+	+	+	+	+
α-ketobutyrate	+	+	+	+		+	+	+	+
α-ketovalerate	+	+	+		+	+		+	+
Propionate								V	
Succinate						+		V	
Alaninamide	+	+	+		+	+	+	+	+
L-alanine	+		+		+	+	V	+	
L-alanylglycine	+					+		V	
L-asparagine	+	+	+		+	+		V	V
L-aspartate								V	
L-glutamate	+	+	+	+	+	+		+	+
Glycyl-L-aspartate								V	
Glycyl-L-glutamate			+					+	
L-histidine								V	
L-leucine	+					+		+	
L-ornithine								V	
L-proline	+	+	+	+	+	+	+	+	+
L-serine	+		+		+	+	+	+	V
L-threonine	V		+	+	+	+	+	+	
Gamma-aminobutyrate								V	
Urocanate	+	+			+	+		+	+

[a] boz = *L. bozemanii* (NCTC 11368), dum = *L. dumoffii* (NCTC 11370), fee = *L. feeleii* (NCTC 12022), hac = *L. hackeliae* serogroup 1 (NCTC 11979), isr = *L. israelensis* (NCTC 12010), lon = *L. longbeachae* serogroup 1 (NCTC 11477), mic = *L. micdadei* (NCTC 11371), pne = pooled results for *L. pneumophila* serogroup 1 (NCTC 11192), 2 (NCTC 11230), 3 (NCTC 11232), 5 (NCTC 11405), 6 (NCTC 11287), 7 (NCTC 11984), 8 (NCTC 11985), 10 (NCTC 12000), 11 (NCTC 12179), 12 (NCTC 12180), 13 (NCTC 12181) and 14 (NCTC 12174), rub = *L. rubrilucens* (NCTC 11987).

[b] + and V denote positive or variable reactions, respectively, in replicate samples.

into a new computer database of *Legionella* species. None of the legionellae tested were identified as closely matching any other of the 434 strains of bacteria in the BIOLOG database, indicating that the profiles obtained were specific to this genus. Environmental isolates which had been identified by conventional means, including serology, as *Legionella* species (kindly provided by Dr. John Kurtz, Oxford) were examined blind using the modified BIOLOG system. The results were compared against a combination of the manufacturer's database and the legionella database constructed in this study. The identification that was obtained agreed with that of Dr. Kurtz, to the species level (Table 3.10a). Unfortunately, all the strains tested in this trial turned out to be *L. pneumophila*. Environmental isolates of *Pseudomonas fluorescens, P. vesicularis, Sphingomonas paucimobilis, Klebsiella spp., Alcaligenes sp., Achromobacter sp.* and other water isolates were not identified as closely matching any of the legionella profiles.

3.4.3 Effect of increased biomass on metabolism

One possible means of encouraging growth and metabolism of microaerophilic bacteria is to increase their cell mass so that the higher respiration rate helps maintain a lower oxygen concentration diffusing into static cultures. When the concentration of the *L. pneumophila* serogroup 1 strain was doubled (approximately 2.3-3.0 x 10^9 cfu ml^{-1}) and the multiwell plates incubated in air, 24 substrates were metabolised and gave positive results within 24 hours (Table 3.9). A similar effect was noted with *L. micdadei*, 18 substrates were metabolised compared with only 9 at the prescribed concentration (data not shown).

3.4.4 Simplification of diluent

The previous experiments described data obtained with legionellae resuspended in PAS since the physiological saline diluent recommended by the manufacturer, which has a sodium chloride concentration of 146 mM, may be inhibitory to some strains (Barbaree *et al.*, 1983). However, PAS is low in sodium (4 mM) and has been shown to provide a good diluent and transport medium for legionellae (Keevil *et al.*, 1989). In an

Table 3.10a Comparison of identification obtained using modified BIOLOG procedure with the results of conventional techniques.

Isolate	Conventional	BIOLOG
K2307	*L. pneumophila* SG9[a]	*L. pneumophila*
K2311	*L. pneumophila* SG9	*L. pneumophila*
K2326	*L. pneumophila* SG1	*L. pneumophila*
K2121	*L. pneumophila* SG1	*L. pneumophila*
K2327	*L. pneumophila* SG1	*L. pneumophila*
K2325	*L. pneumophila* SG1	*L. pneumophila*
K4275	*L. pneumophila* SG1	*L. pneumophila*
K2697	*L. pneumophila* SG1	*L. pneumophila*
K2344	*L. pneumophila* SG6	*L. pneumophila*

[a]SG = serogroup.

attempt to simplify this diluent, which also contains 1 mM K^+, 2 mM phosphate, 27 μM Ca^{2+} and 16 μM Mg^{2+}, legionellae were resuspended and incubated in deionised water. This was carried out with bacterial suspensions double of that described in the manufacturer's manual. Using this methodology similar results were obtained with deionised water as diluent as were obtained using PAS.

Employing the latter two modifications, similar profiles were obtained for the strains investigated as were recorded in the initial experiments using a low oxygen incubation and PAS as diluent. Sufficient numbers of positive reactions could be obtained within 24 hours, using distilled water as diluent and in a normal aerobic atmosphere, to allow accurate identification of the strains tested. Thus the modified protocol was considerably simplified for routine laboratory use. However, the profiles obtained differed in some reactions, therefore it would be necessary to construct an independent database for use with the modified procedure.

The BIOLOG experiments demonstrate that legionellae are metabolically versatile and the possibility that they can be identified biochemically.

3.5 PLANKTONIC BACTERIAL GROWTH IN THE EVAPORATIVE COOLING TOWER DURING VARIOUS MODES OF OPERATION

3.5.1 *Legionella pneumophila*

The cooling tower was successfully operated in a number of different regimes designed to simulate a variety of operating conditions which might be encountered in cooling towers under field conditions. These operating conditions each resulted in different levels of *L. pneumophila* growth.

3.5.1a Mode 1: Spring/Autumn cooling demand

The first mode of operation (Mode 1), *Spring/Autumn* cooling demand, resulted in high

densities of *L. pneumophila* in the cooling tower water (Figure 3.6). The concentration of *L. pneumophila* in the water was 1000 CFU l^{-1} immediately after inoculation. This fell to 250 CFU l^{-1} by 5 days post-inoculation but then increased significantly to 2.6 x 10^5 CFU l^{-1} by day 11. Legionellae numbers reached a maximum of 8.75 x 10^5 CFU l^{-1} 18 days after inoculation, before stabilising at around 4.7 x 10^5 CFU l^{-1} 31 days post-inoculation and remaining at this level for the rest of the run (a further 14 days). The mean temperatures during this mode of operation were 23.8°C and 31.0°C in the pond and heat exchanger, respectively.

3.5.1b Mode R1: Modified repeat of Mode 1

Mode 1 experiment was repeated after modifications had been made to the system (as described in section 2.1.3 b). This repeat experiment was designated Mode R1. The modifications were designed to allow the system to more closely mimic operational cooling towers. The cooling tower was able to operate at the required number of cycles of concentration. It was, however, necessary to increase the air flow through the tower to ensure correct operation of the fail-safe system which was installed in the intake duct (see Section 2.4.1a). The initial concentration of *L. pneumophila* in the system immediately after inoculation was 1125 CFU l^{-1}, similar to levels in Mode 1. The cooling tower was operated in a similar fashion, except that the system operated at higher cycles of concentration and the increased air flow resulted in temperature differences. The mean temperatures in the pond and heat exchanger were 21.3°C and 25.9°C, respectively.

This modified repeat of *Spring/Autumn* demand resulted in intermittent isolation of *L. pneumophila* with counts obtained through the first 35 days of the experiment varying from no legionellae isolated (*i.e.* below the 100 CFU l^{-1} detection level) to counts of 100 to 300 CFU l^{-1}. An increase in the concentration of *L. pneumophila* occurred after day 35. Counts obtained were in the region of 1 x 10^4 to 2 x 10^4 CFU l^{-1} (Figures 3.7 and 3.18).

Two other repeats of Mode 1 were carried out as it was considered that conditions which yielded further high *L. pneumophila* counts would provide valuable data for correlation analysis. These repeats were subject to the same constraints as Mode R1 and were similar in outcome (data not shown).

3.5.1c Mode 2: Continuous steady demand during the working day

The second mode of operation (Mode 2), *Continuous steady demand during the working day*, involved continuous operation of the tower for 8 hours per day with the pump and fan active throughout this period (fan speed was 2.5 m s^{-1}). The size of the inoculum was similar to that used previously, however the concentration of *L. pneumophila* detectable in the system immediately after inoculation was 100 CFU l^{-1}. *L. pneumophila* was not isolated from samples collected 24 hours after inoculation nor from samples collected daily for 5 weeks subsequently (Figure 3.8). Mean water temperature in the pond and heat exchanger were 21.1 and 24.5°C, respectively.

3.5.1d Mode R2 (Repeat of Mode 2)

Figures 3.9 and 3.13 show the results obtained during a repeat of Mode 2 of operation (referred to as Mode R2). A larger inoculum was used to ensure initial colonisation. The initial concentration of *L. pneumophila* in the cooling tower was 2300 CFU l^{-1}, this decreased to 100 CFU l^{-1} over a 24 hour period and persisted at 100 to 200 CFU l^{-1} until day 10. No legionellae were isolated from the system for the rest of the run after day 10. The mean water temperatures in the pond and heat exchanger were 22.8 and 25.4°C, respectively.

3.5.1e Mode 3: (Intermittent demand)

The third mode (Mode 3) of operation used was *Intermittent demand.* An initial concentration of 1000 CFU l^{-1} of *L. pneumophila* was detected in the system subsequent to inoculation. Intermittent demand resulted in *L. pneumophila* persisting in the system for an extensive period (78 days) at between 200 an 2200 CFU l^{-1}, only falling below

detection level (< 100 CFU l^{-1}) in samples collected subsequent to a period of tower inactivity which occurred 54 days after inoculation (Figures 3.10 and 3.18). The mean *L. pneumophila* count up until this point was 686 CFU l^{-1}. During this mode of operation the mean water temperatures in the pond and heat exchanger were 22.5 and 25.2°C, respectively.

3.5.2 Heterotrophic bacteria in the cooling tower during various modes of operation

3.5.2a Mode 1

Total bacterial counts (expressed as heterotrophic plate counts, HPC) followed a similar trend to *L. pneumophila* counts during Mode 1 operation (Figure 3.6).

3.5.2b Mode R1

The heterotrophic plate count was relatively constant through out Mode R1 operation (6 x 10^8 - 8 x 10^8 CFU l^{-1}) (Figure 3.7).

3.5.2c Mode 2

The total heterotrophic bacterial count in the water during Mode 2 was higher than during any of the other experiments (Figure 3.8 and 3.18).

3.5.2d Mode R2

The mean total heterotrophic count during this repeat of Mode 2 was around 4-fold lower than in the initial experiment using this continuous mode of operation (Figure 3.9 and 3.18) but was similar to that obtained during Mode R1.

3.5.2e Mode 3

Even although Mode 3 was carried out immediately after Mode 2, with no disinfection or draining between the different modes of operation, the mean total heterotrophic plate count was around a third of that obtained during Mode 2 (Figure 3.10).

The cooling system was open to contamination from both the air and the make-up water, therefore, a variety of bacteria other than legionellae were isolated from the cooling tower. Only the bacteria which were consistently isolated were considered to be capable of colonising the system. Isolates which fell into this category were investigated further, others were simply counted and recorded. Six colony types were consistently isolated. These were all Gram negative bacteria. It is widely recognised that identification of heterotrophs from water is no simple matter (Wadowsky & Yee, 1983; Stout *et al.*, 1985; Toze *et al.*, 1990), and for that reason the identifications, where obtained, should not be treated as being definitive. Consequently the isolates are simply referred to as HB1, HB2, HB3, HB4, HB5 and HB6. Differentiation was carried out on the basis of colony morphology and microscopic examination of Gram stained preparations. HB1 was identified as *Pseudomonas vesicularis*; HB2 as *Pseudomonas* sp., HB3 as *Pseudomonas stutzeri*, HB4 *Aeromonas* sp. Identifications were not achieved for the other two isolates, however they both formed pigmented colonies on R2A agar (HB5 was orange and HB6 was pink). On the basis of mean counts it was determined that HB3 was the most numerous member of the heterotrophic population throughout all experiments irrespective of how the tower was operated. HB3 made-up 59% of the HPC in Mode 1; 48.5% in Mode 2; 53% in Mode R1; 62% in Mode R2; and 46% in Mode 3. The profile of the heterotroph population for each mode of operation are shown in Figures 3.11 - 3.15.

The only significant correlation between the heterotrophic bacteria isolated and *L. pneumophila* was a negative correlation between the concentrations of HB5 and *L. pneumophila* in the cooling tower water (a product-moment correlation coefficient, r of -0.6279 at $P = 0.000$). HB5 colony type accounted for 25% of the heterotrophic population during Mode 2 and was essentially absent from Modes 1, Mode R1 and Mode R2. This colony type persisted in the system, albeit at a lower concentration (mean count for Mode 3 of 6%), throughout Mode 3. This mode of operation followed directly on from Mode 2 with no disinfection between these operating regimes.

There was no significant correlation between *L. pneumophila* concentration and the total number of heterotrophs in the system at the $P < 0.05$ level. However, if a slightly less rigorous criterion was applied there was a small negative correlation between *L. pneumophila* numbers and the heterotroph count (a product-moment correlation coefficient, r of -0.1830 at $P = 0.063$.

Figure 3.6 Bacterial counts in cooling tower water during "Spring/Autumn" mode of operation.

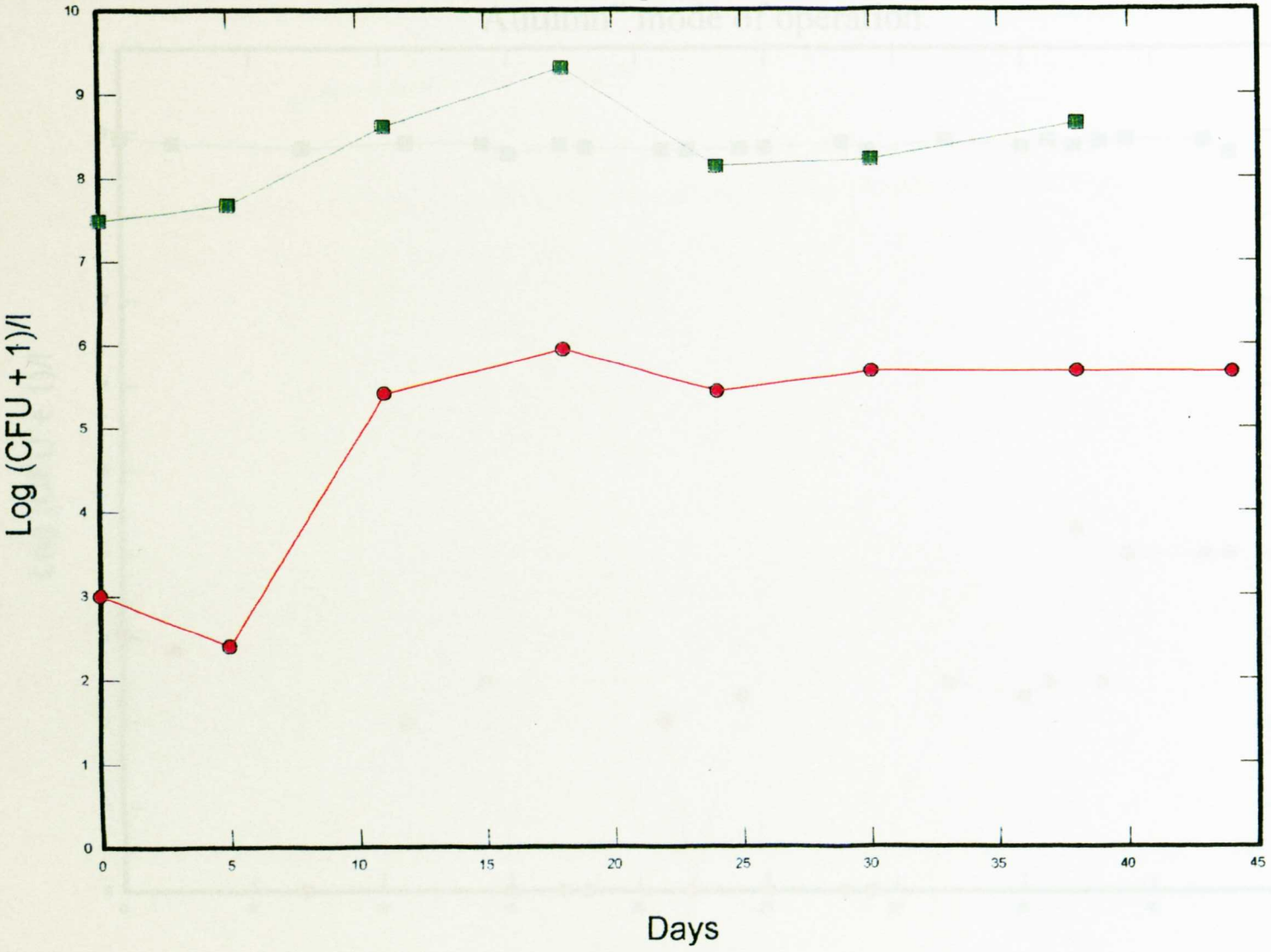


Figure 3.7 Bacterial counts in cooling tower water during partial repeat of "Spring/ Autumn" mode of operation.

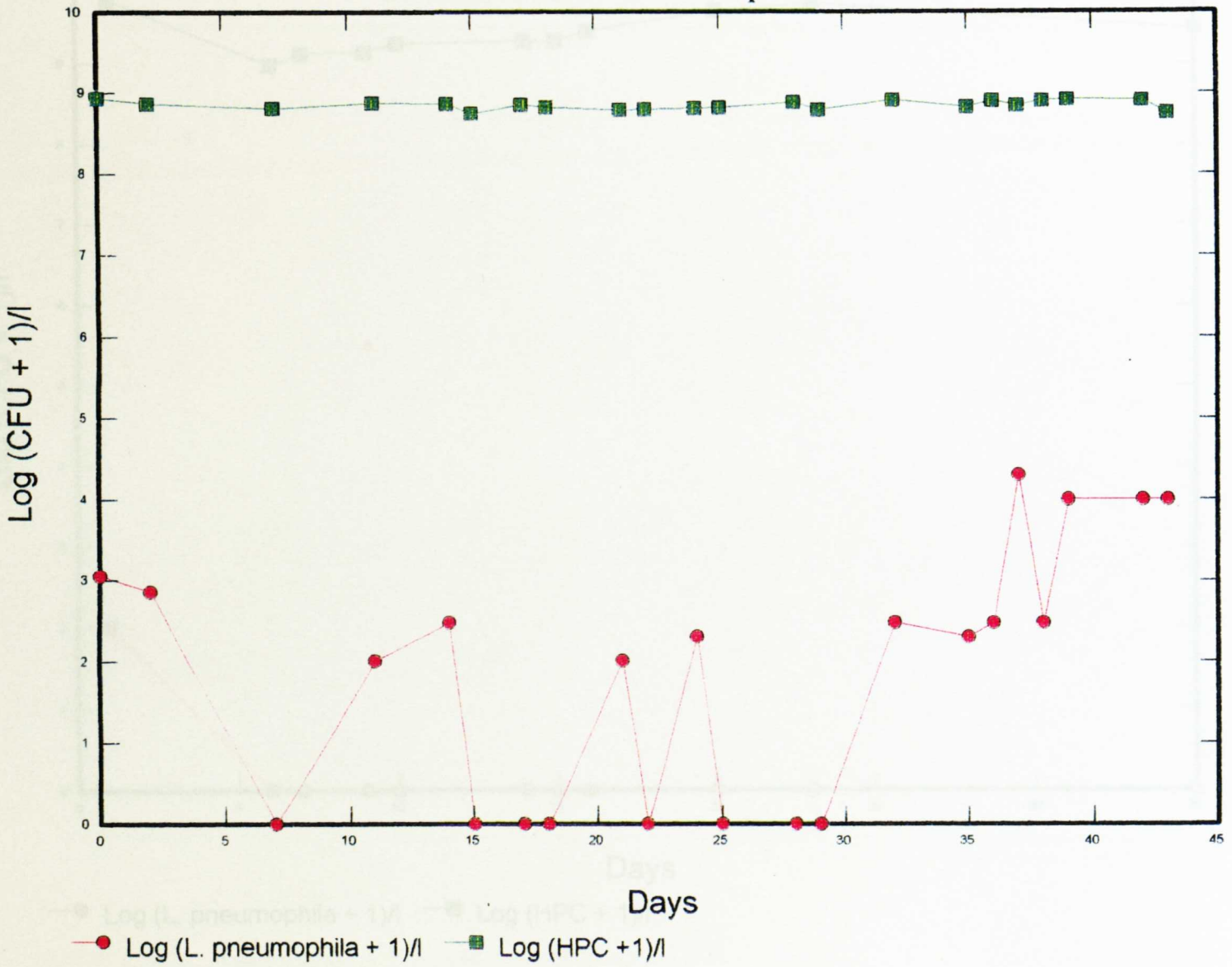


Figure 3.8 Bacterial counts in cooling tower water during continuous operation through "working day".

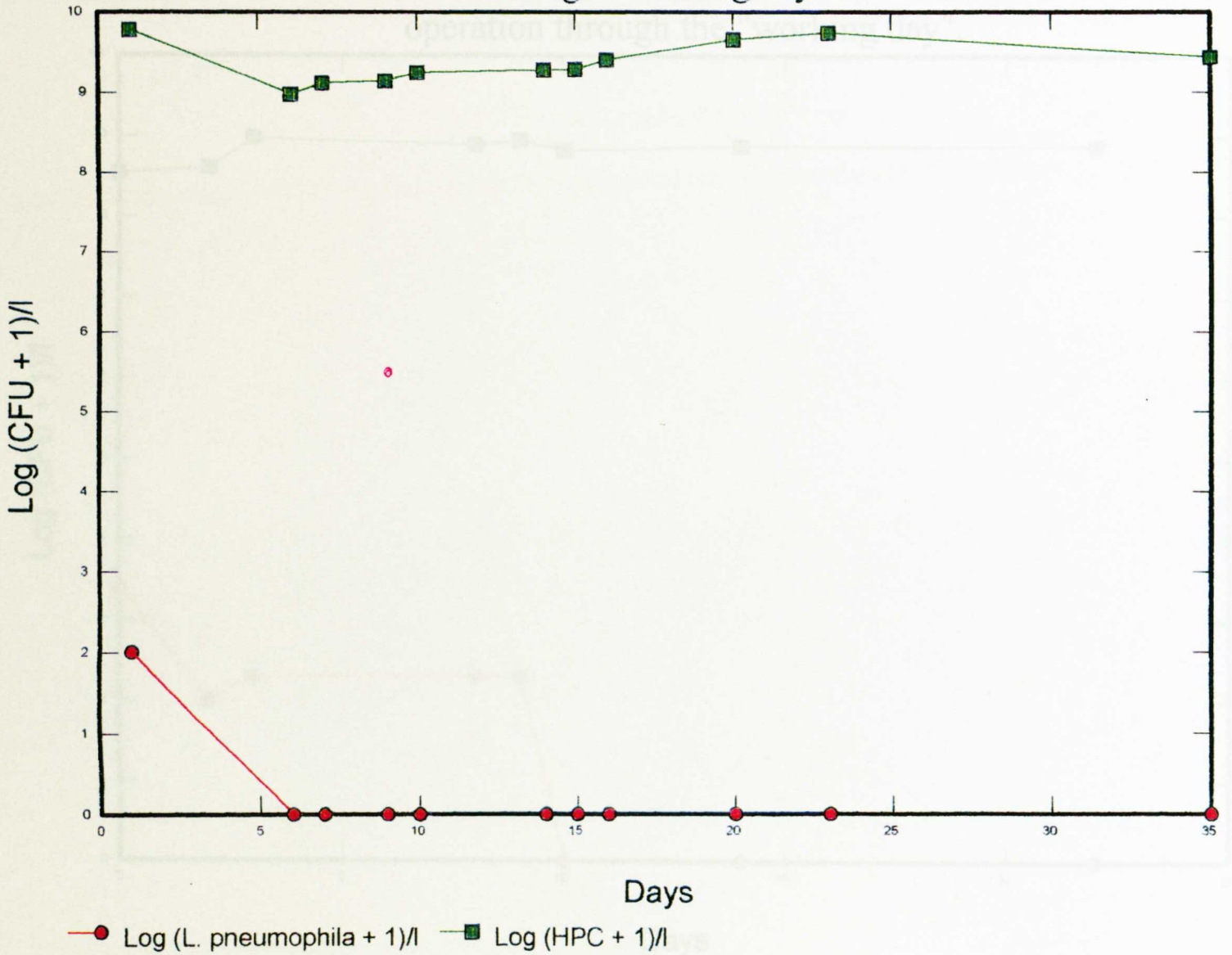


Figure 3.9 Bacterial counts in cooling tower water during repeat of continuous operation through the "working day".

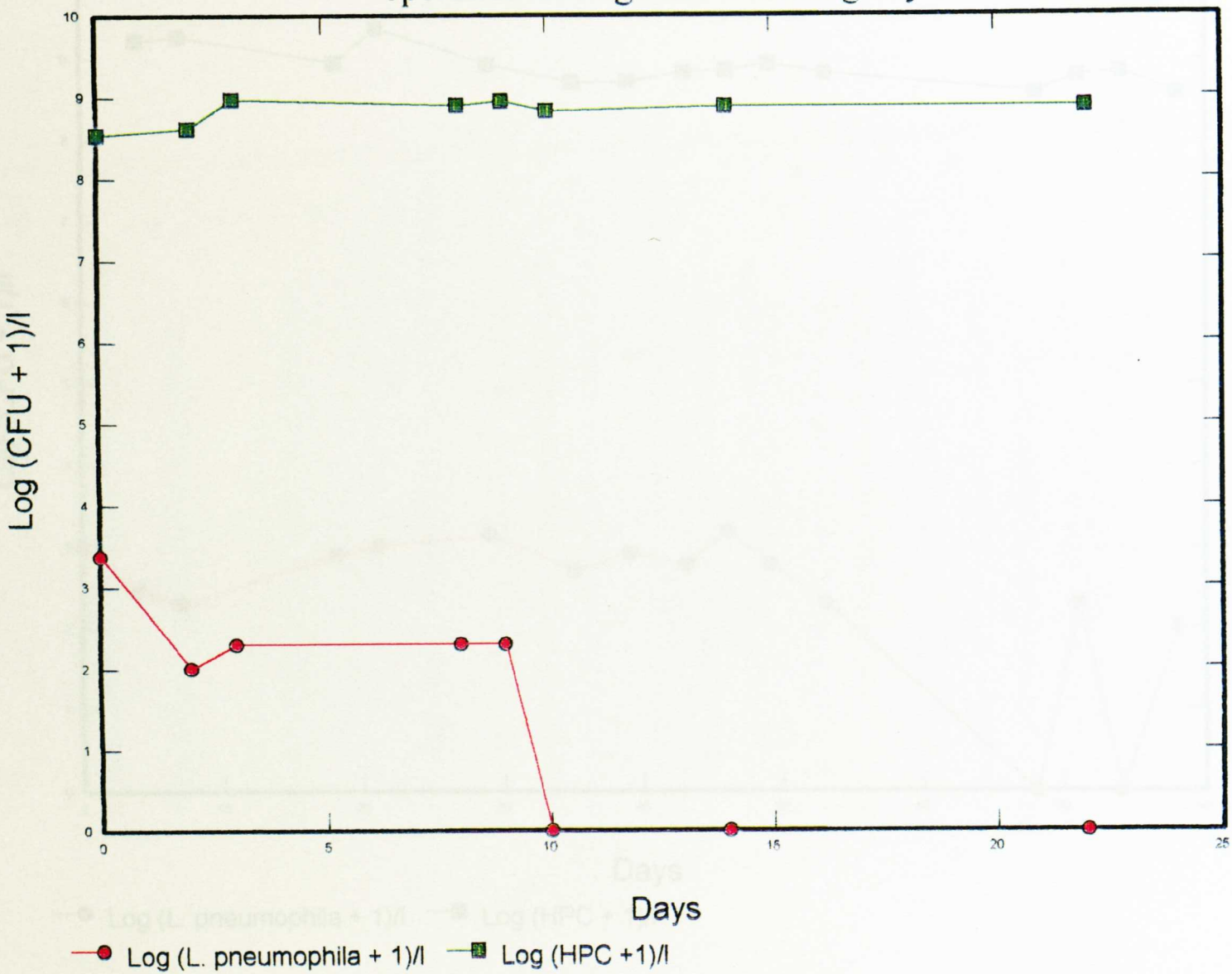


Figure 3.10 Bacterial counts in cooling tower water during intermittent operation.

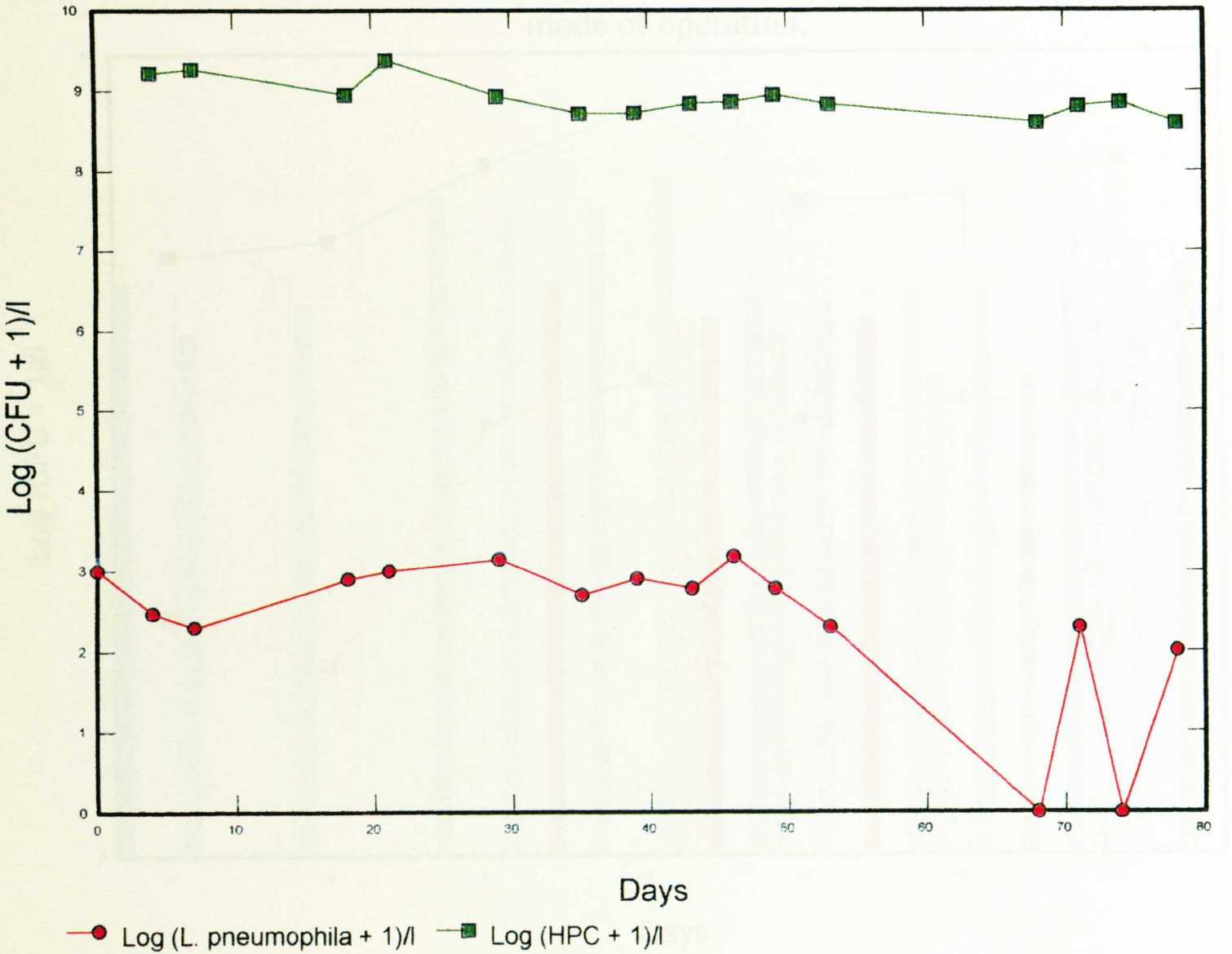


Figure 3.11 Heterotrophic bacteria in cooling tower water during "Spring Autumn" mode of operation.

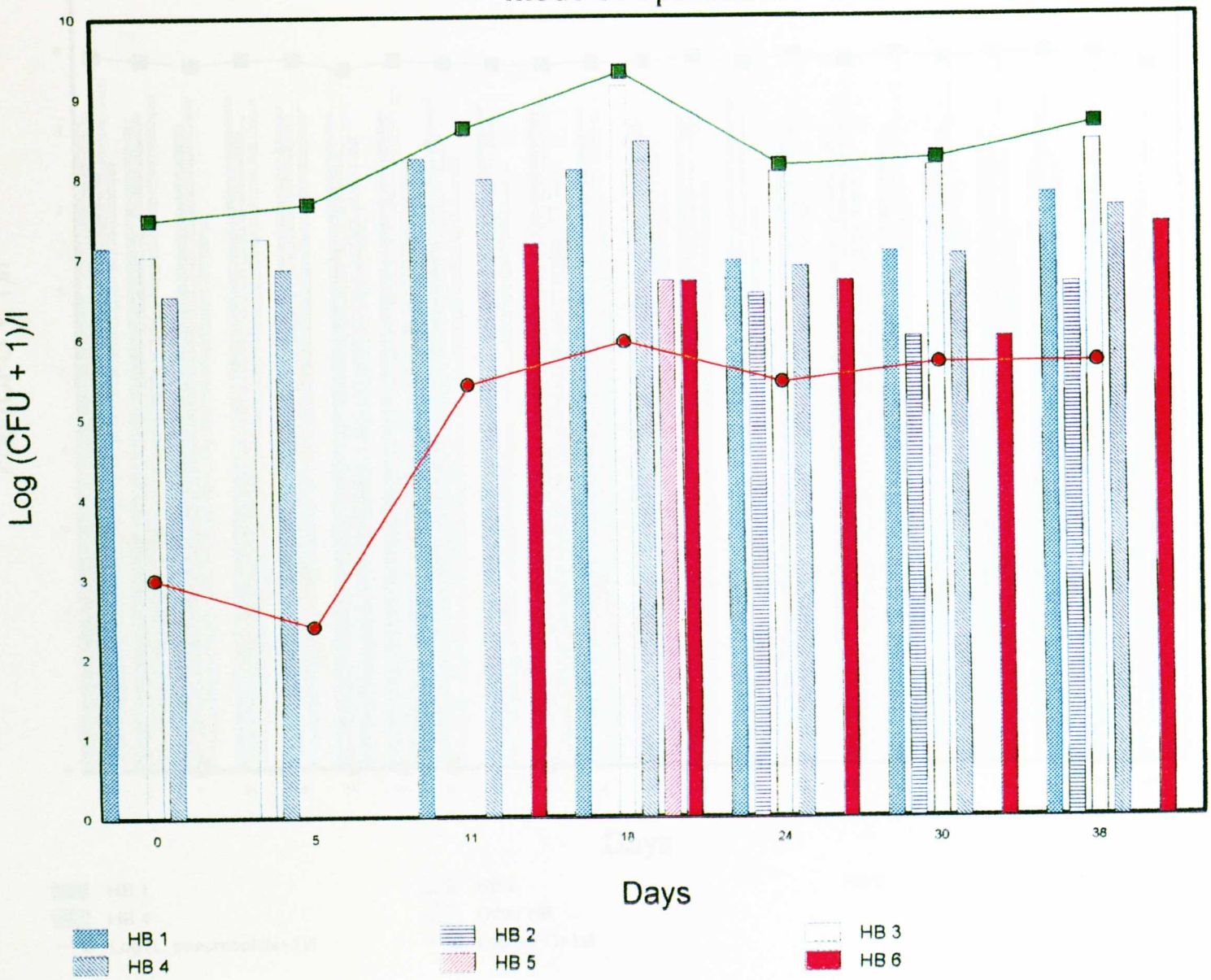


Figure 3.12 Heterotrophic bacteria in cooling tower water during partial repeat of "Spring Autumn" mode of operation.

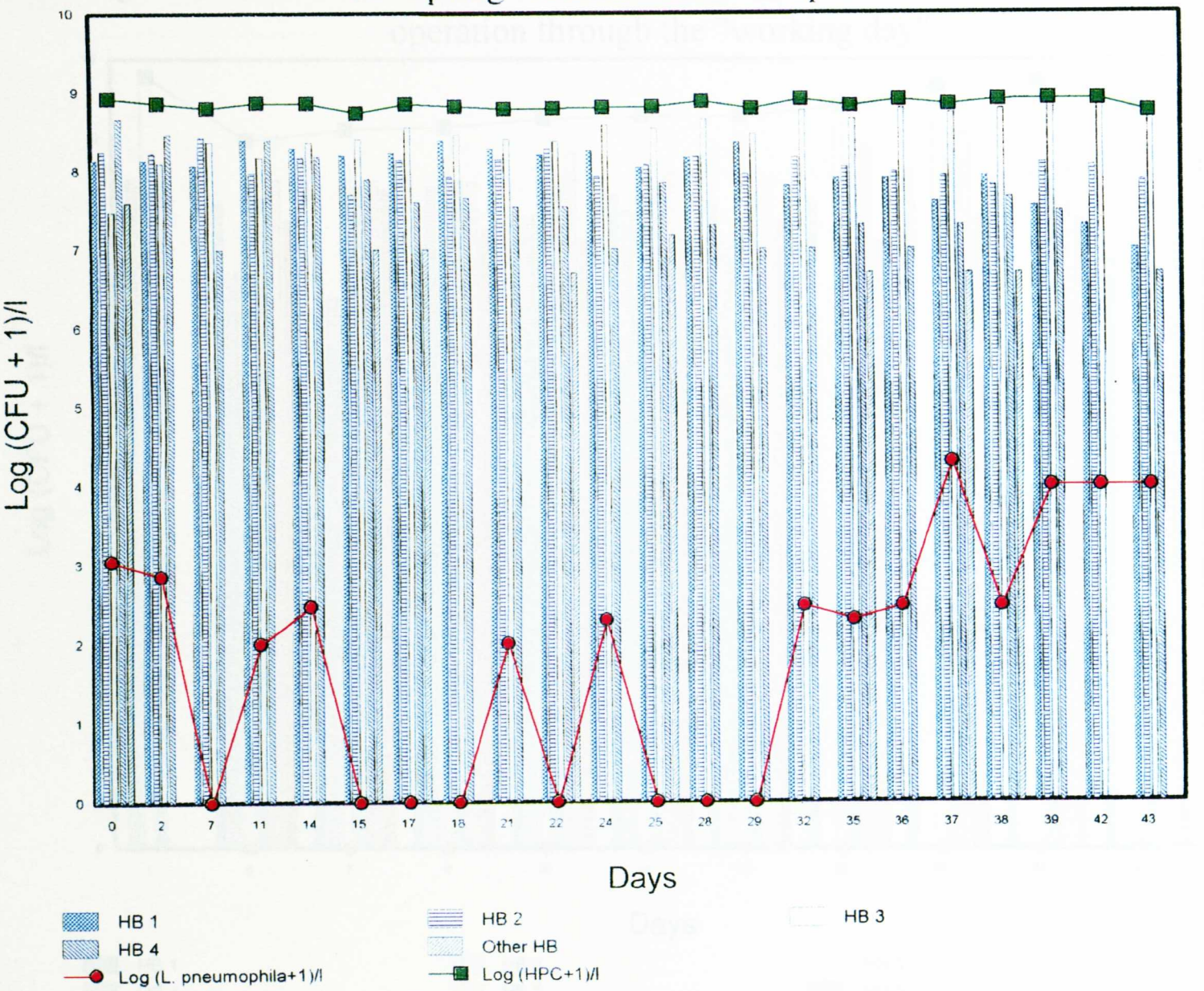


Figure 3.13 Heterotrophic bacteria in cooling tower water during continuous operation through the "working day".

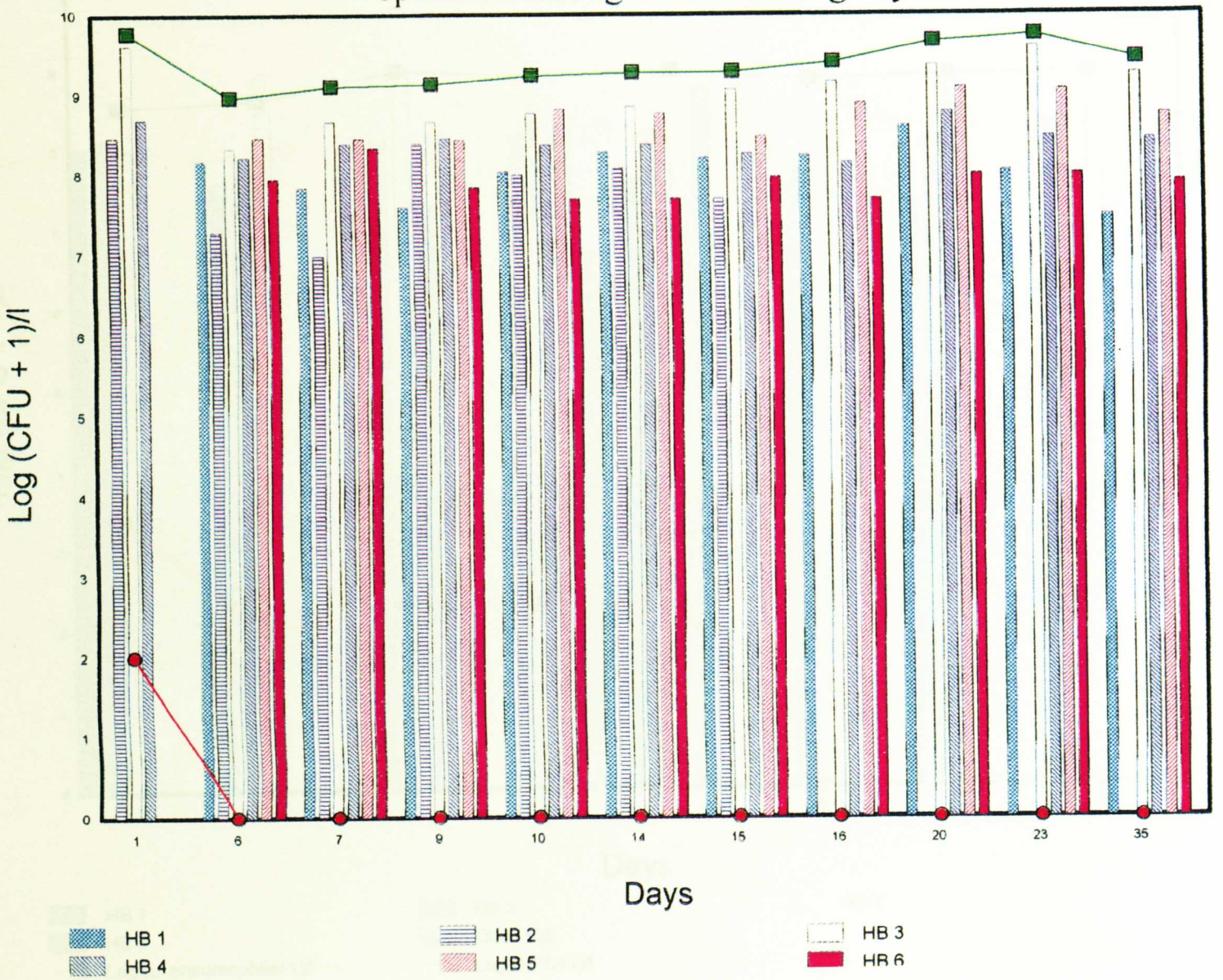


Figure 3.14 Heterotrophic bacteria in cooling tower water during partial repeat of continuous operation through the "working day".

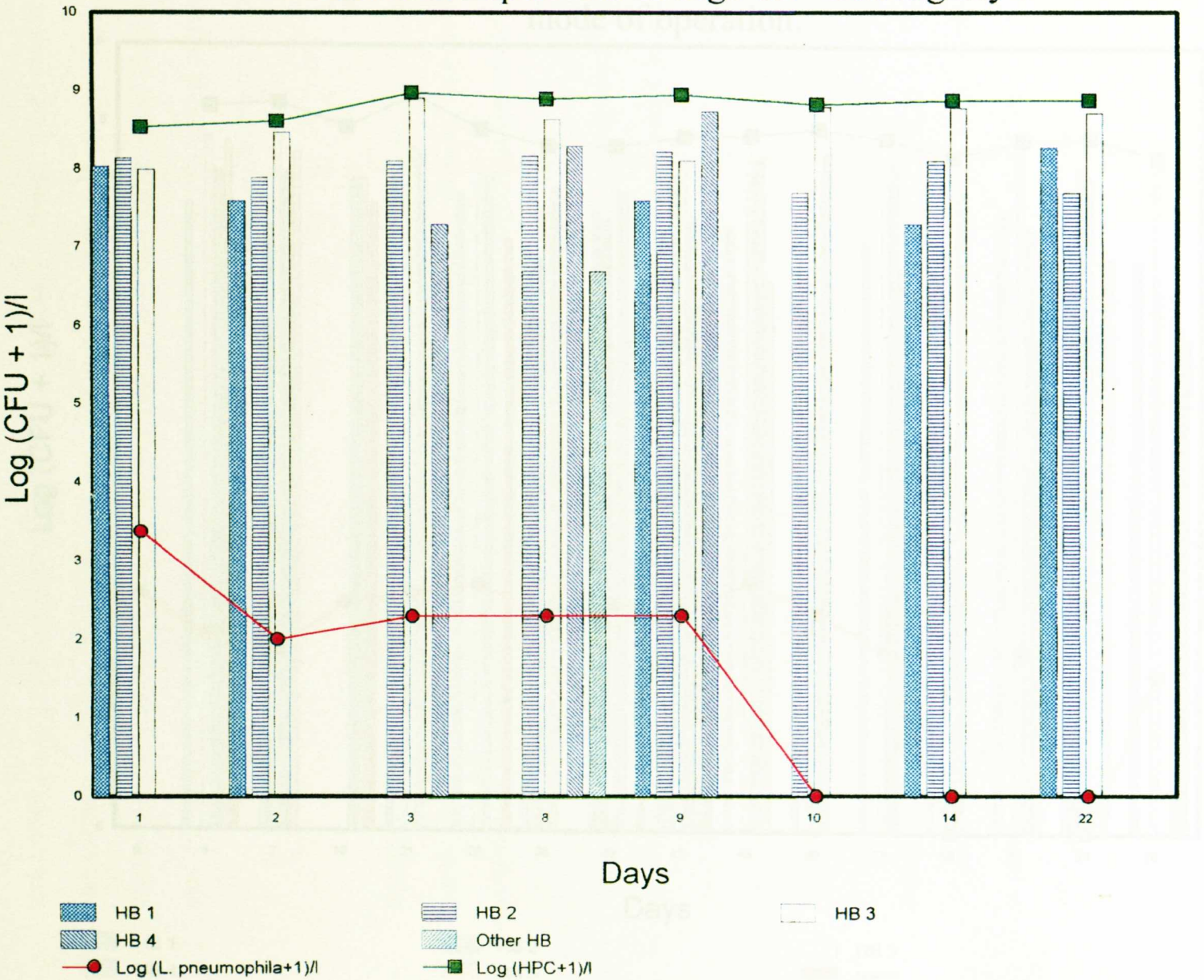
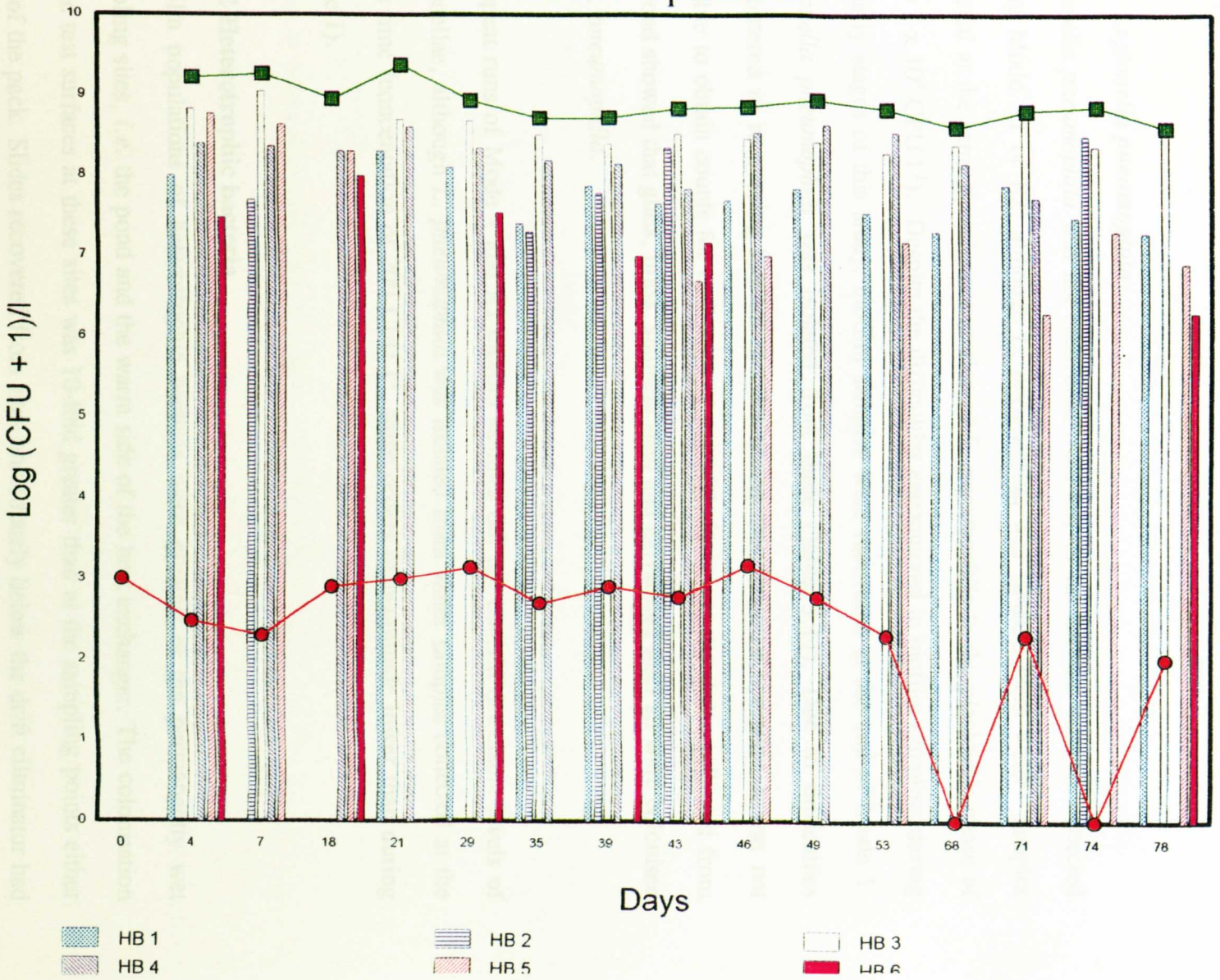


Figure 3.15 Heterotrophic bacteria in cooling tower water during intermittent mode of operation.



3.6 SURFACE ASSOCIATED GROWTH WITHIN THE SYSTEM

Satisfactory recovery of regular biofilm samples from the system was only achieved during Mode R1 and Mode R2.

3.6.1 *Legionella pneumophila*

Legionella pneumophila was not recovered from any of the biofilm samples collected during Mode R1 or R2, although *L. pneumophila* was isolated from water samples collected at the same time (concentration of *L. pneumophila* in the planktonic phase of up to 2 x 10^4 CFU l^{-1}). Despite the difficulties encountered in biofilm sampling during the early stages of this study, biofilm samples were recovered at the end of Mode 1. *Legionella pneumophila* was isolated from these (Table 3.11). Due to difficulties encountered in recovering biofilm samples during this mode of operation it was not possible to obtain counts for each type of material, however, the slides collected from the pond showed that glass, brass, stainless steel and galvanised steel all were colonised by *L. pneumophila.*

In repeat runs of Mode 1 and R1 biofilm samples did not contain detectable levels of legionellae, although *L. pneumophila* was isolated from water samples collected at the same time (concentation of 200 CFU l^{-1}, three orders of magnitude lower than during Mode 1).

3.6.2 Heterotrophic bacteria

Biofilm populations of heterotrophic bacteria were greatest at the permanently wet sampling sites, *i.e.* the pond and the warm side of the heat exchanger. The colonisation of the test surfaces at these sites was 10-fold greater than at the sampling points either side of the pack. Slides recovered from sites immediately below the drift eliminator had significantly less bacterial colonisation (Figures 3.16 and 3.17). The heterotrophic biofilm populations in the pond and heat exchanger remained relatively constant

Table 3.11 *Legionella pneumophila* colonisation of surfaces in the cooling tower at the end of *Spring/Autumn* mode of operation.

Site	Material	*L. pneumophila* (x 10^5 CFU m^{-2})
Pond	Glass	9.5
	Brass	3.2
	Stainless steel	6.3
	Galvanised steel	6.3
Heat exchanger	Brass	BDL
	Stainless steel	9.5
	Galvanised steel	BDL
Below pack	Glass	3.1
Above pack	Glass	3.1
Drift eliminator	Glass	BDL

[a] BDL = below detection level of 1.667 x 10^5 CFU m^{-2}.

Figure 3.16 Heterotrophic bacteria in biofilm samples during partial repeat of "Spring Autumn" mode of operation.

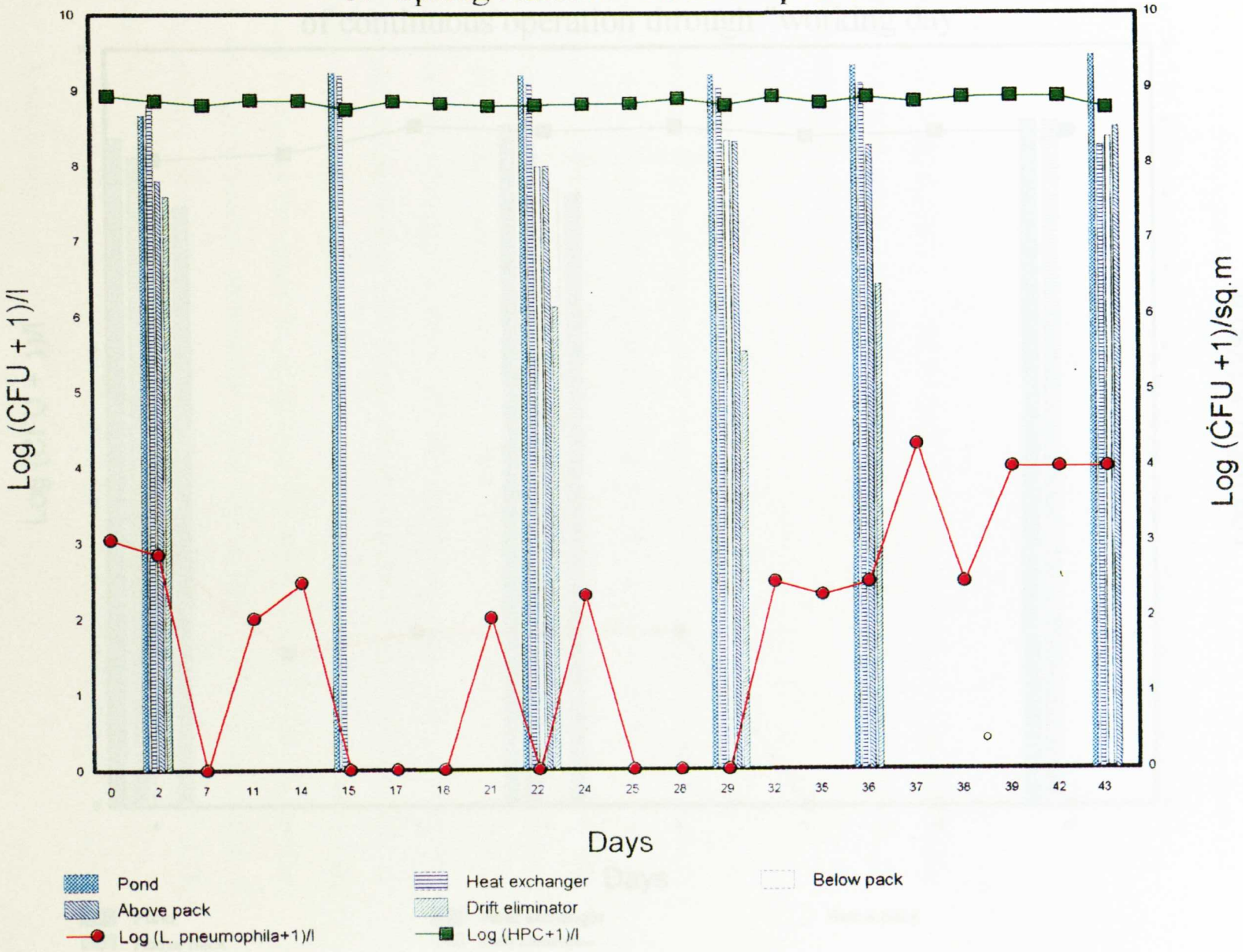
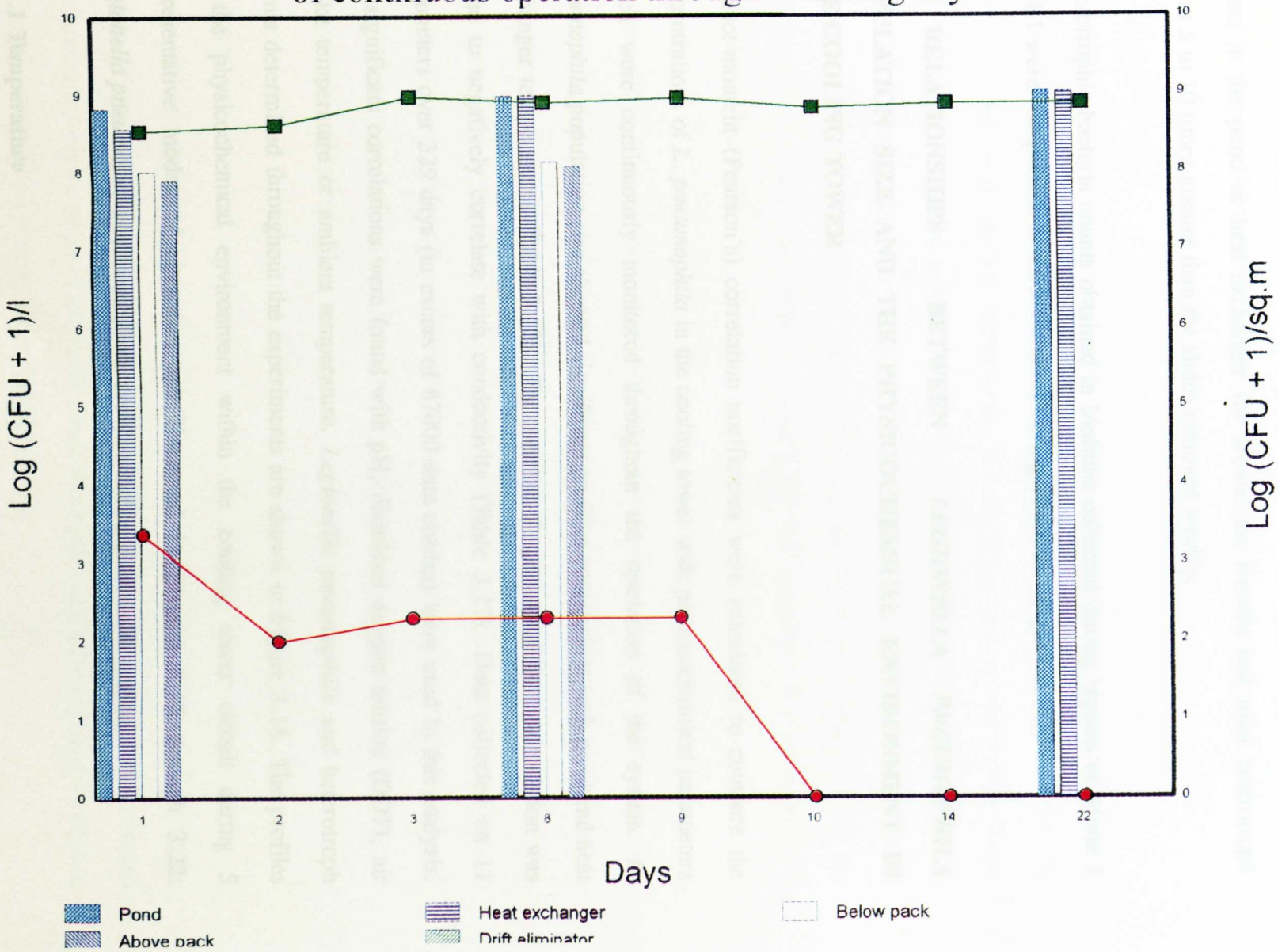


Figure 3.17 Heterotrophic bacteria in biofilm samples during partial repeat of continuous operation through "working day".



throughout Mode R1 and Mode R2. The data presented in Figures 3.16 and 3.17 represent bacterial populations after 7 days of biofilm development. Slides which were present in the pond or heat exchanger for around six months had total heterotroph counts 5 to 10 times greater than the slides removed weekly.

Heterotrophic bacteria counts obtained in biofilms collected during repeats of Mode 1 and R1 were comparable to those obtained during Mode R1 (Figure 3.16).

3.7 RELATIONSHIPS BETWEEN *LEGIONELLA PNEUMOPHILA* POPULATION SIZE AND THE PHYSICOCHEMICAL ENVIRONMENT IN THE COOLING TOWER

Product-moment (Pearson's) correlation coefficients were calculated to compare the concentrations of *L. pneumophila* in the cooling tower with physicochemical parameters which were continuously monitored throughout the operation of the system. *L. pneumophila* population size showed significant positive correlations with pond and heat exchanger temperatures (Table 3.12). By contrast, *L. pneumophila* concentration was found to negatively correlate with conductivity (Table 3.12). Data collected on 11 parameters over 329 days (in excess of 87000 data entries) were used in this analysis. No significant correlations were found with pH, dissolved oxygen tension (DOT), air intake temperature or ambient temperature. *Legionella pneumophila* and heterotroph counts determined throughout the experiments are shown in Figure 3.18. The profiles of the physicochemical environment within the cooling tower circuit during 5 representative modes of operation are illustrated in Figures 3.19 through 3.22. *Legionella pneumophila* concentrations are also included on these graphs.

3.7.1 Temperature

The mean pond temperature during Mode 1, the operating regime which resulted in the highest concentrations of *L. pneumophila* in the system was 23.8°C. This temperature

was higher than that of the other modes of operation (by 1 - 2.5°C). How ever, the mean heat exchanger temperature (31°C) was ≥ 5°C higher than temperatures recorded during the other modes of operation (Figure 3.14).

3.7.2 Conductivity

Legionellae numbers were greatest when conductivity was lowest (mean value of 0.72 mS cm^{-1}) *i.e.* during Mode 1. The mean conductivity during Mode 2 was 1.450 mS cm^{-1} calculated from on-line monitoring; this corresponded to legionellae being undetectable in the cooling tower. The cycles of concentration were increased during Mode R2 to investigate the effect of increasing the total dissolved solid (TDS) concentration in the recirculating water (TDS is proportional to conductivity) on the population size of *L. pneumophila*. Increased TDS did not alter the concentration of legionellae in Mode R2.

Due to failure of the conductivity cell during Mode 3 incomplete on-line data was obtained for this period and so this parameter was omitted from the portion of Figure 3.20 pertaining to this period. Off-line measurements were obtained from water samples collected at regular intervals during this mode of operation and the conductivity was controlled accordingly. Although conductivity was similar during Mode 2 and Mode 3 (mean value of 1.480 mS cm^{-1}), legionellae were isolated from Mode 3 but not during Mode 2 (Figure 3.20).

3.7.3 pH

There was no correlation between the concentration of *L. pneumophila* and pH in the system. The pH range was 8.2 to 9.2. A damaged probe during Modes 2 and 3 resulted in inaccurate measurements of pH in the pond, these were therefore replaced by off-line pH determinations and omitted from Figure 3.21.

Table 3.12 Significant correlations ($P < 0.05$) between numbers of *Legionella pneumophila* and physicochemical parameters in the cooling tower.

Parameter	r[a]	Probability[b]
Conductivity	-0.5006	0.000
Heat exchanger temperature	0.3861	0.000
Pond temperature	0.2987	0.000

[a] Product-moment correlation coefficient.

[b] Probability of occurring by chance.

Figure 3.18 Bacteria in cooling tower water through all modes of operation.

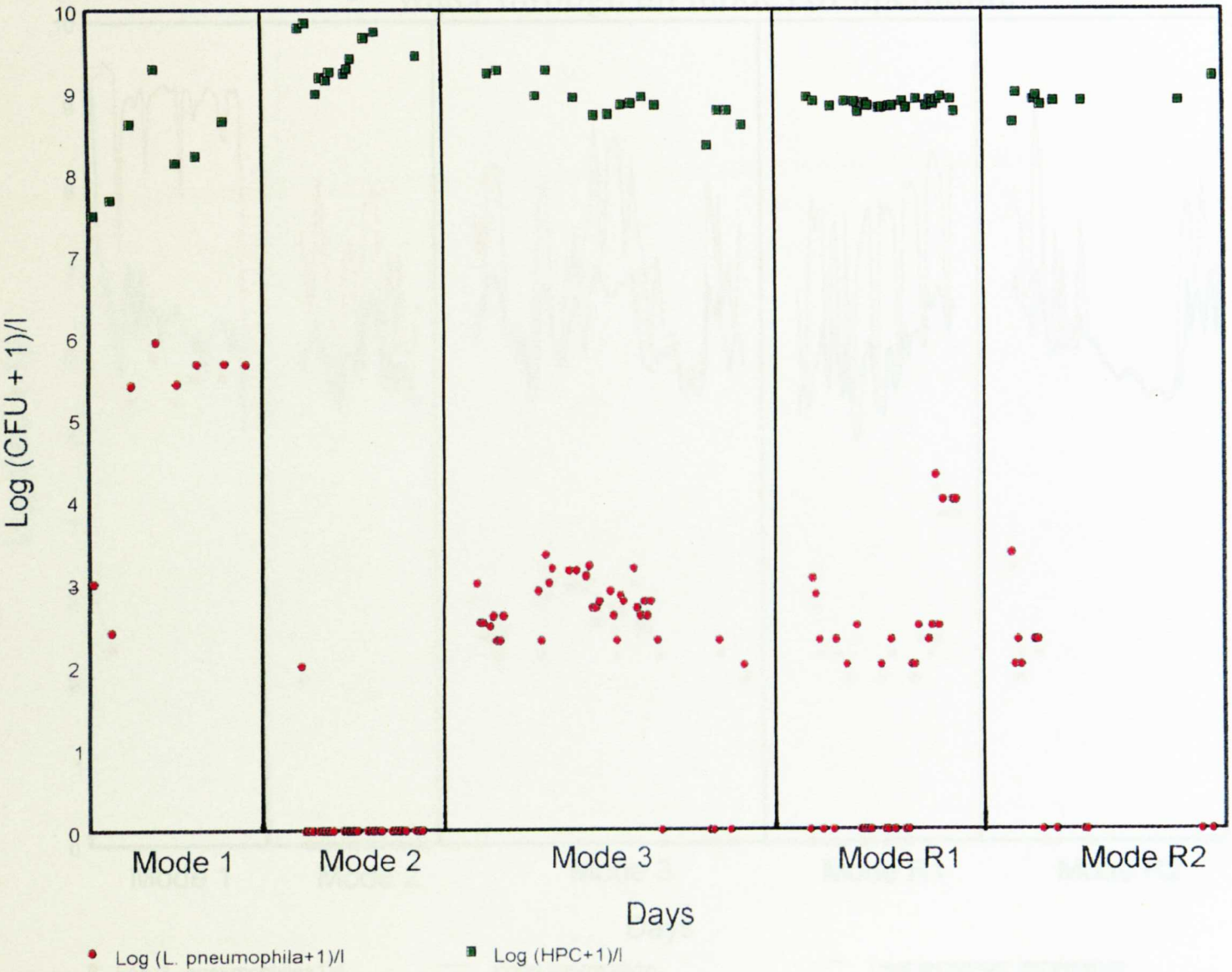


Figure 3.19 Legionella pneumophila and temperatures in cooling tower water through all modes of operation.

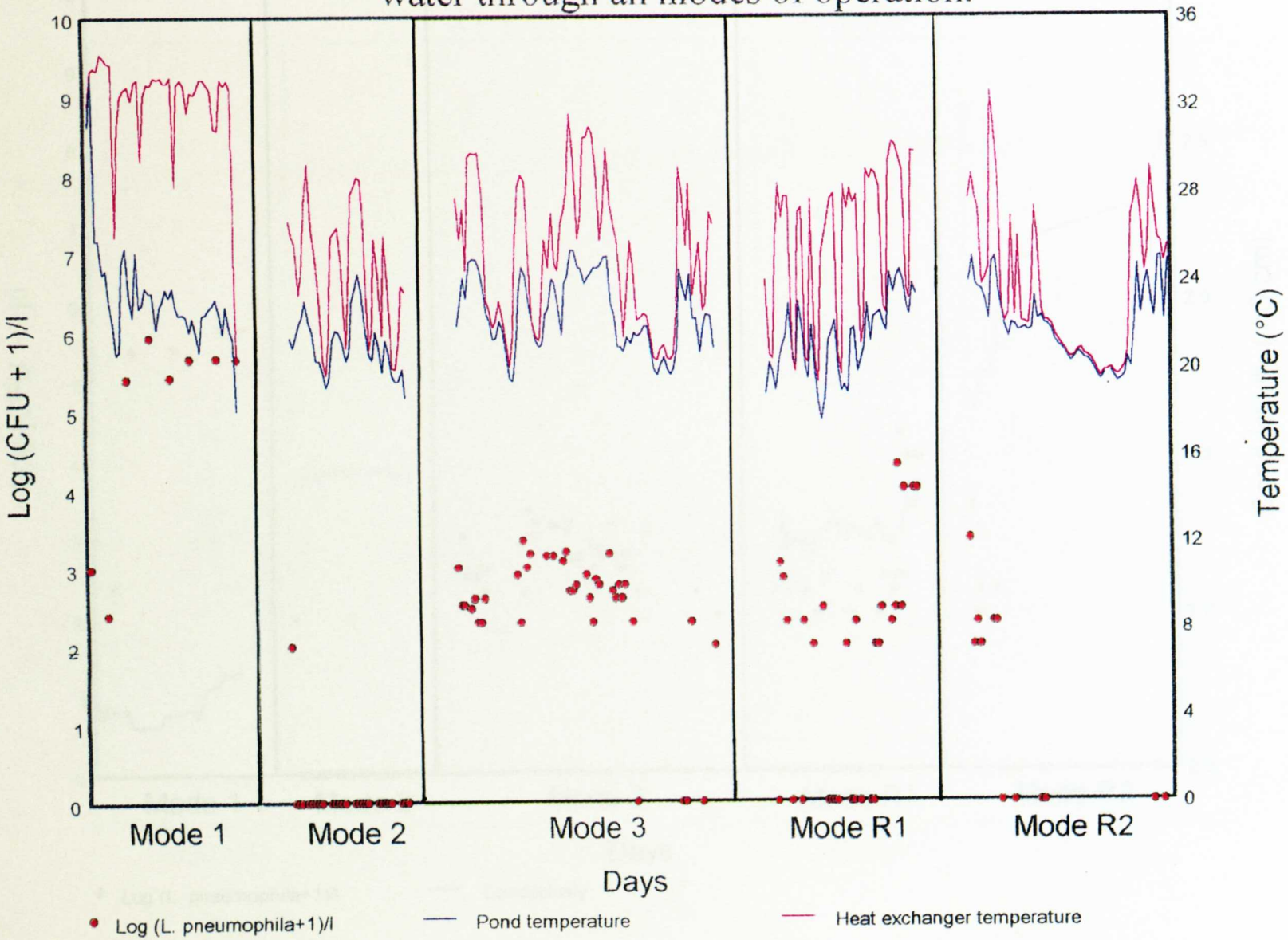


Figure 3.20 Legionella pneumophila and conductivity in cooling tower water through all modes of operation.

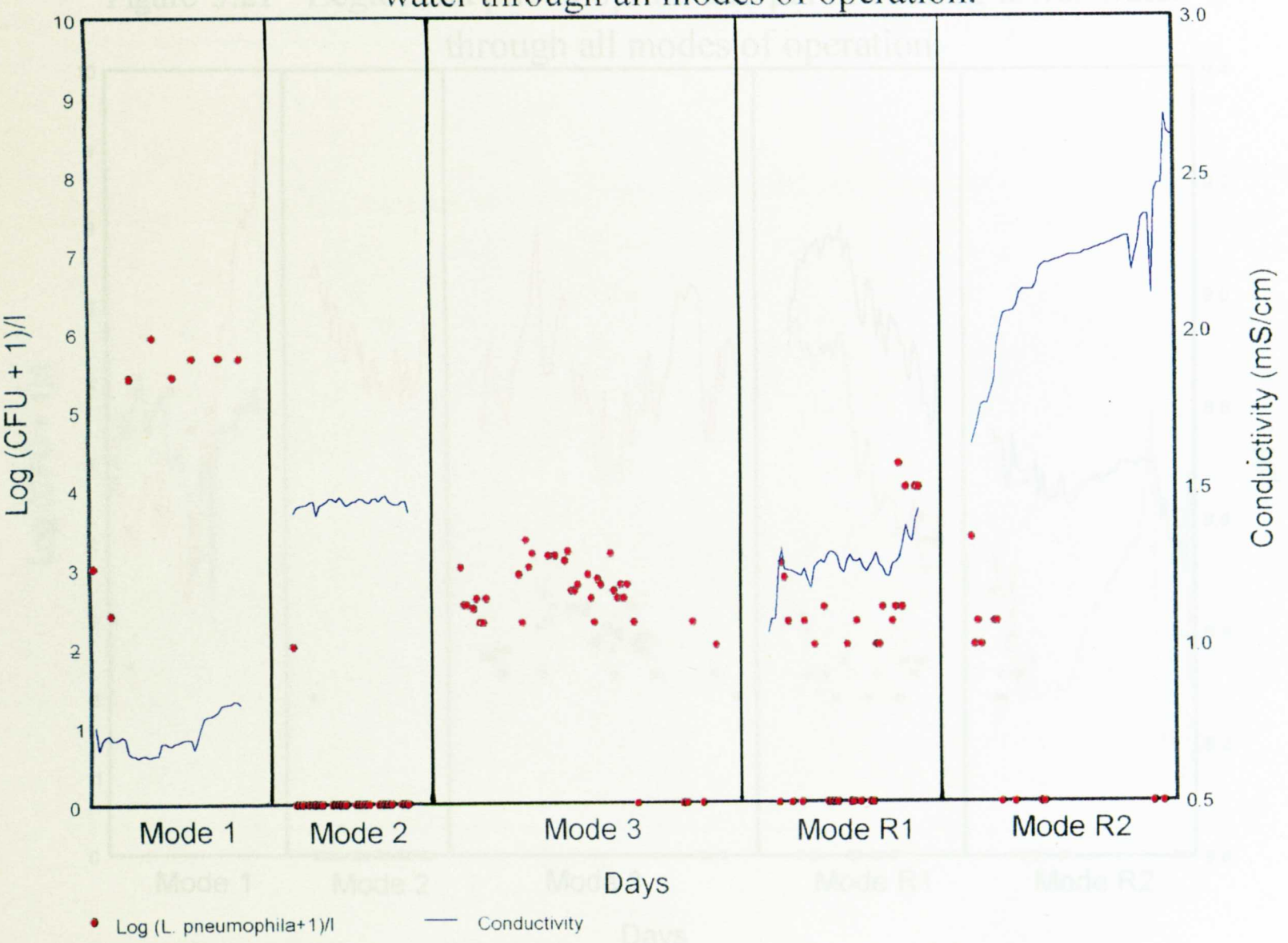


Figure 3.21 Legionella pneumophila and pH in cooling tower water through all modes of operation.

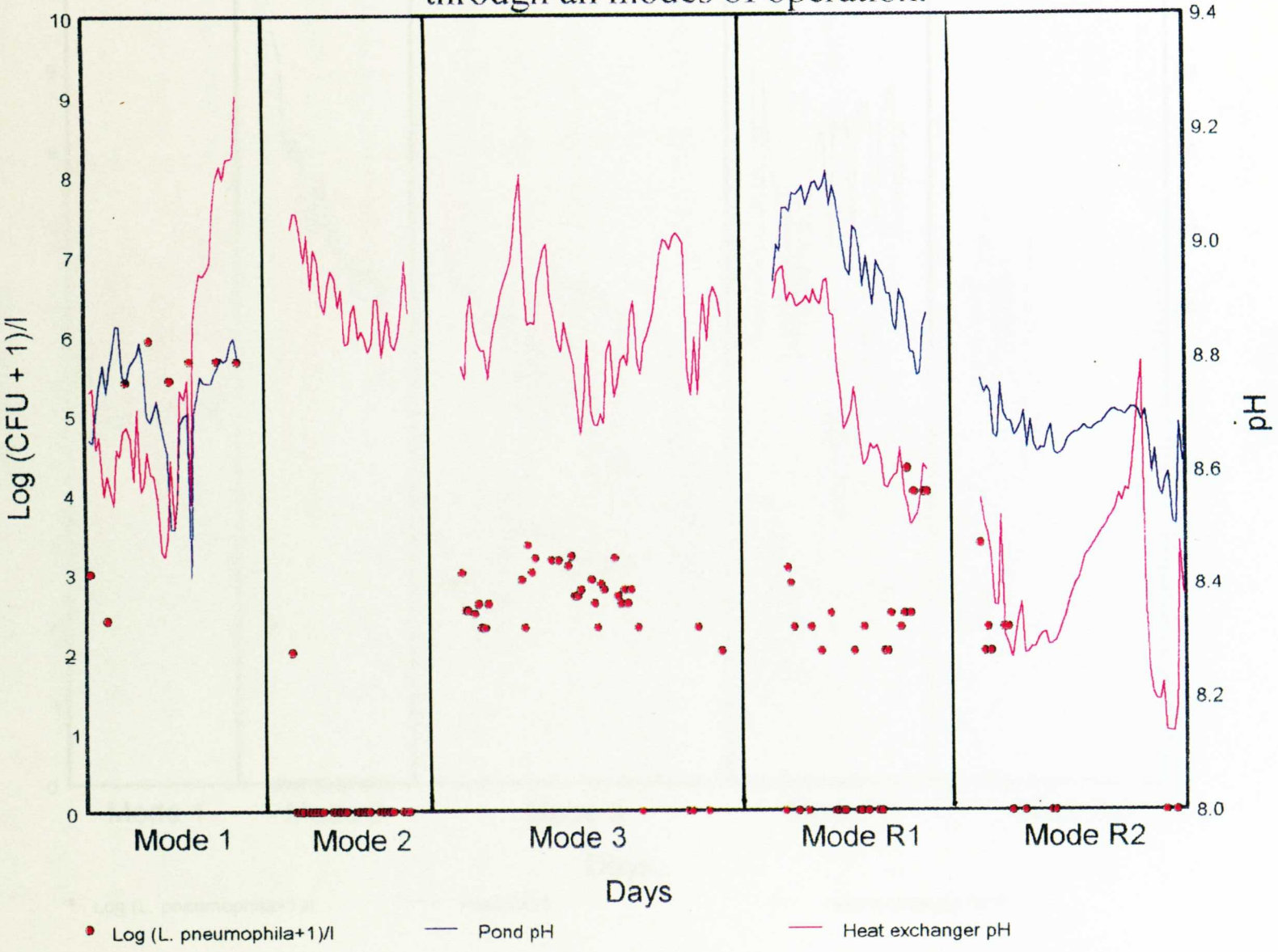
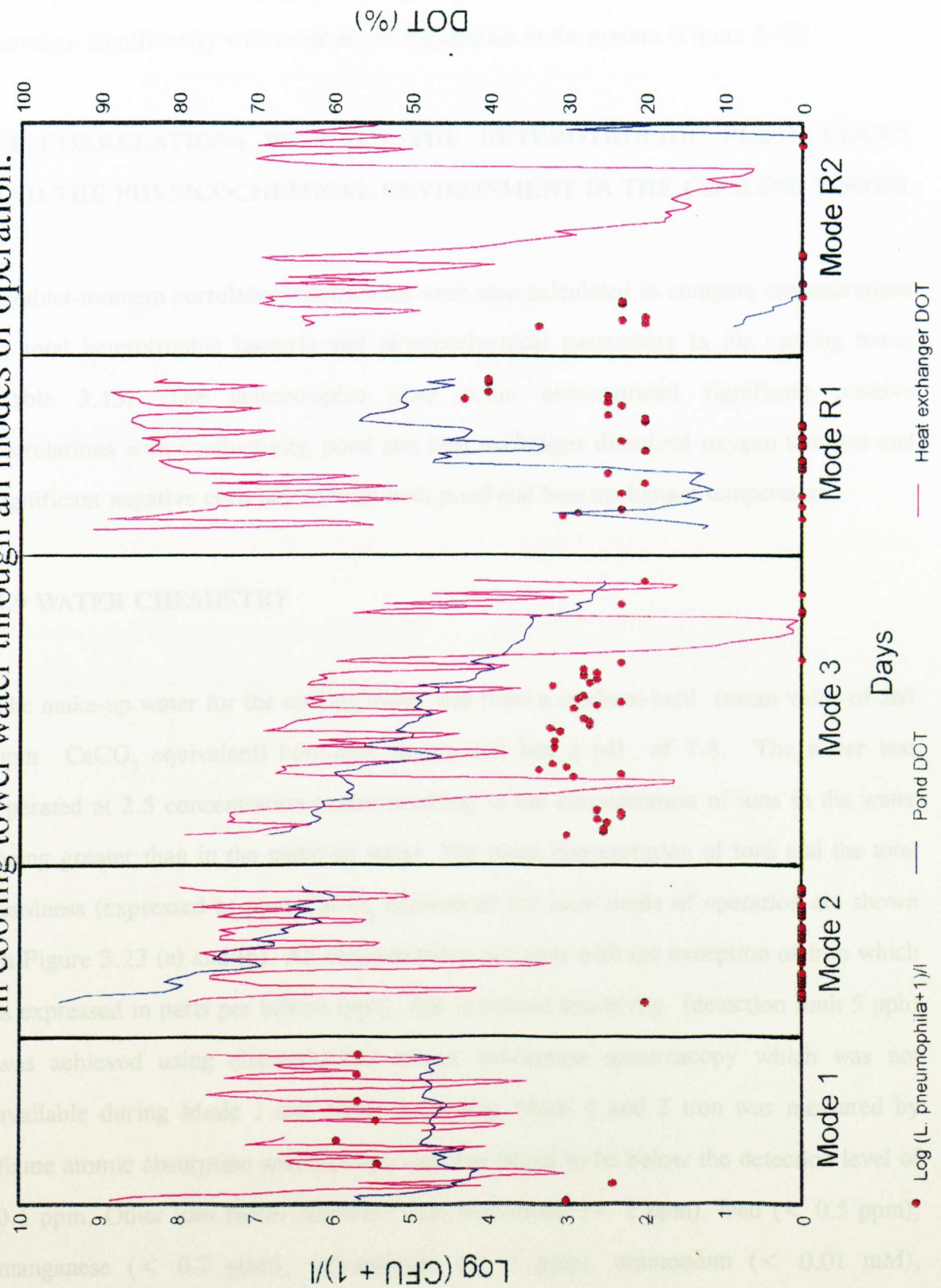


Figure 3.22 Legionella pneumophila and dissolved oxygen tension (DOT) in cooling tower water through all modes of operation.



3.7.4 Dissolved oxygen tension

This parameter varied greatly throughout the experiments but was not found to correlate significantly with numbers of legionellae in the system (Figure 3.22).

3.8 CORRELATIONS BETWEEN THE HETEROTROPHIC PLATE COUNT AND THE PHYSICOCHEMICAL ENVIRONMENT IN THE COOLING TOWER

Product-moment correlation coefficients were also calculated to compare concentrations of total heterotrophic bacteria and physicochemical parameters in the cooling tower (Table 3.13). The heterotrophic plate count demonstrated significant positive correlations with conductivity, pond and heat exchanger dissolved oxygen tensions and significant negative correlations with both pond and heat exchanger temperatures.

3.9 WATER CHEMISTRY

The make-up water for the cooling tower was from a medium-hard (mean value of 267 ppm $CaCO_3$ equivalent) bore-hole supply and had a pH of 7.8. The tower was operated at 2.5 concentration cycles resulting in the concentration of ions in the water being greater than in the make-up water. The mean concentration of ions and the total hardness (expressed as ppm $CaCO_3$ equivalent) for each mode of operation are shown in Figure 3.23 (a) and (b). All concentrations are ppm with the exception of iron which is expressed in parts per billion (ppb), this increased sensitivity (detection limit 5 ppb) was achieved using electrothermal atomic absorption spectroscopy which was not available during Mode 1 and Mode 2. During Mode 1 and 2 iron was measured by flame atomic absorption spectroscopy and was found to be below the detection level of 0.1 ppm. Other ions below detection were aluminium (< 2 ppm), lead (< 0.5 ppm), manganese (< 0.2 ppm), molybdenum (< 1 ppm), ammonium (< 0.01 mM), phosphate (< 0.1 ppm). Copper was detected in some samples (range 0.12 to 0.31 ppm) but was below detection in the majority of samples. Total solids and total organic

Table 3.13 Significant correlations ($P < 0.05$) between numbers of heterotrophic plate count bacteria and physicochemical parameters in the cooling tower.

Parameter	r[a]	Probability[b]
Conductivity	0.3579	0.002
Heat exchanger DOT	0.2228	0.027
Pond DOT	0.4457	0.000
Heat exchanger temperature	- 0.4039	0.000
Pond temperature	- 0.3124	0.003

[a] Product-moment correlation coefficient.

[b] Probability of occurring by chance.

Figure 3.23(a) Chemical analysis of cooling tower water through all modes of operation

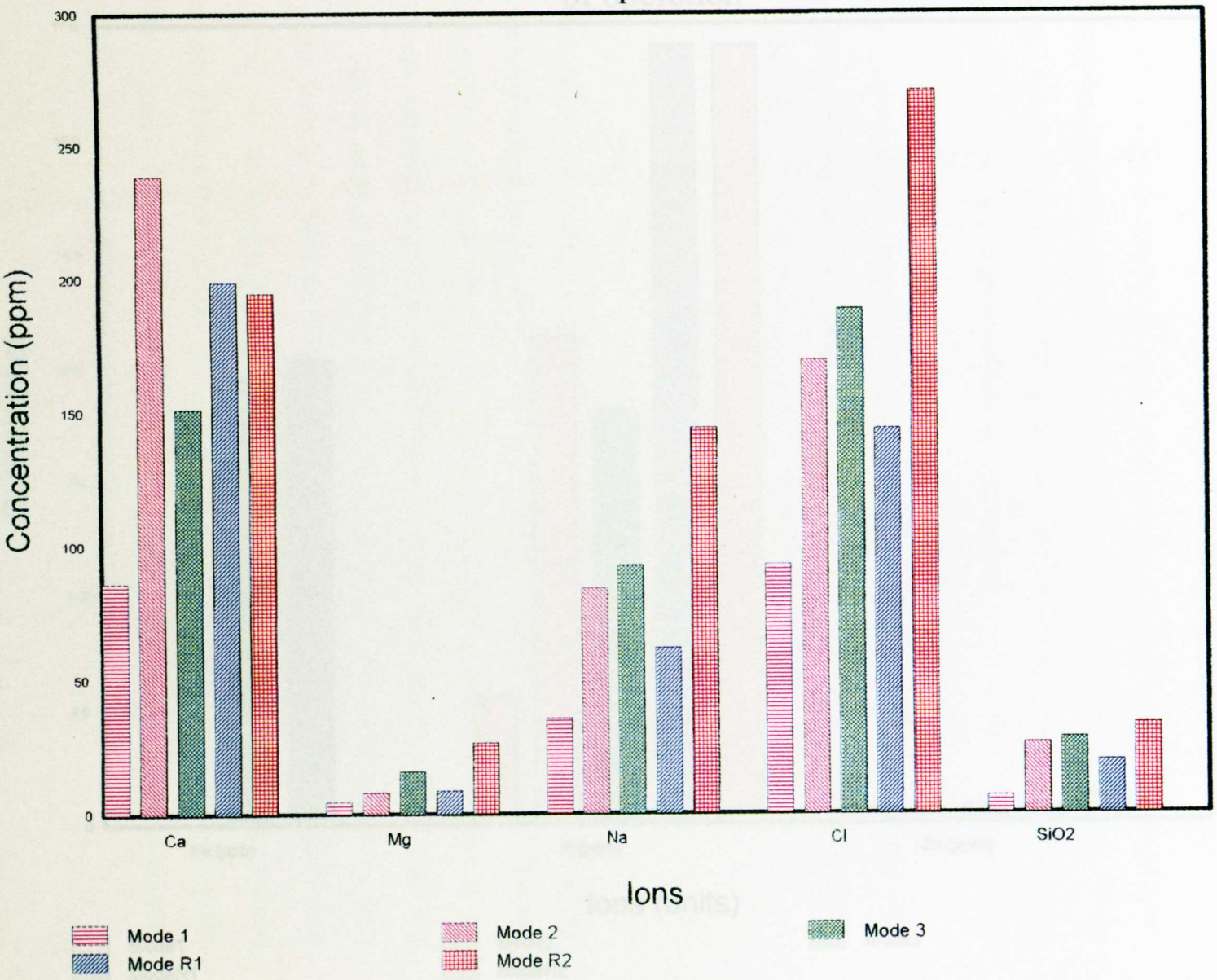
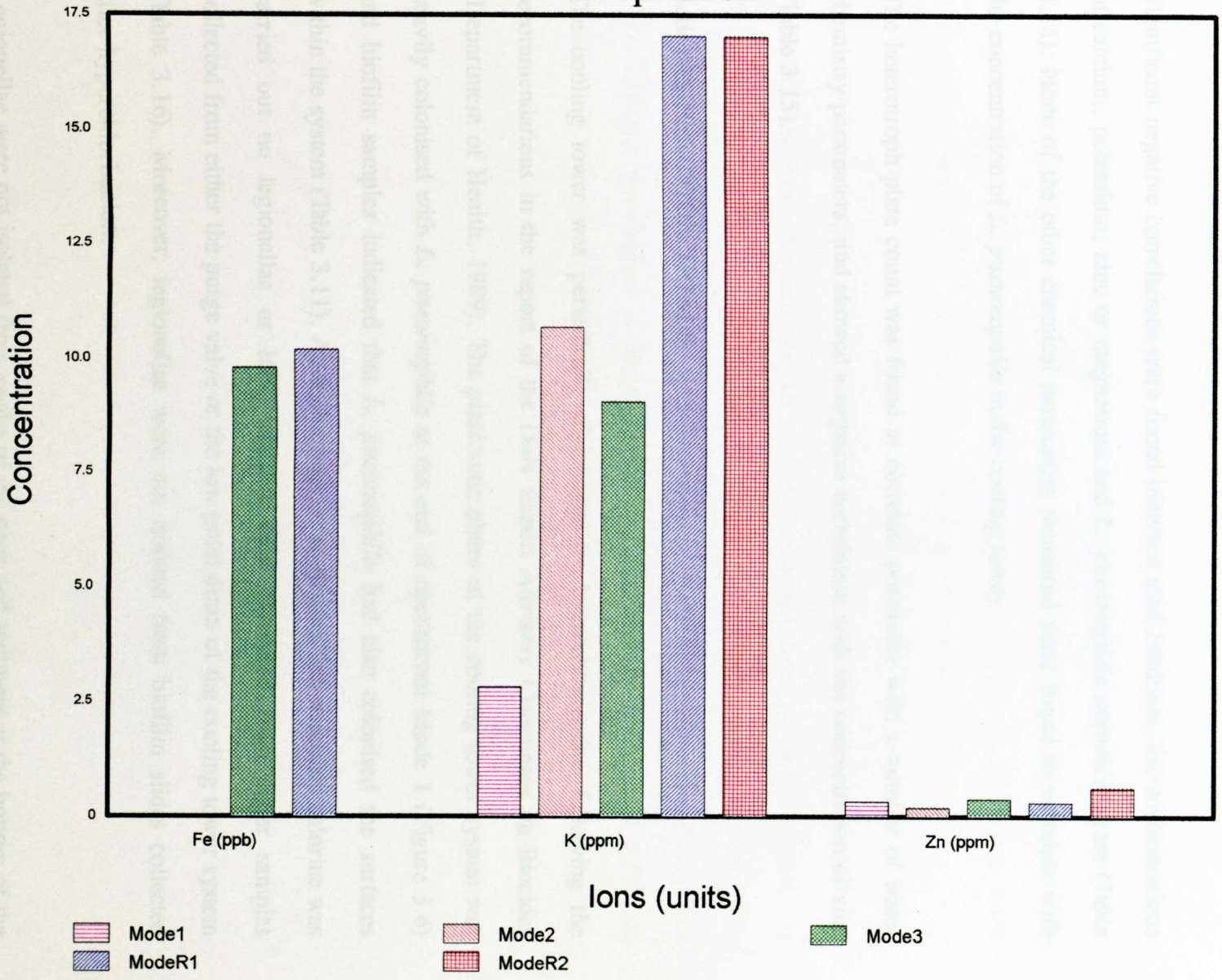


Figure 3.23 (b) Chemical analysis of cooling tower water through all modes of operation



carbon (TOC) analyses were only available during Mode R1 and Mode R2. The mean concentration of total solids was 1102 and 1483 ppm for Mode R1 and Mode R2, respectively. Mean TOC values for Modes R1 and Modes R2 were 10.1 and 17.5 ppm, respectively.

Significant negative correlations were found between total hardness, the concentrations of calcium, potassium, zinc or magnesium and *L. pneumophila* population size (Table 3.14). None of the other chemical parameters measured were found to correlate with the concentration of *L. pneumophila* in the cooling tower.

The heterotroph plate count was found to correlate positively with a number of water chemistry parameters, and showed a negative correlation with the concentration of zinc (Table 3.15).

3.10 DISINFECTION TRIALS

The cooling tower was periodically disinfected by hyperchlorination following the recommendations in the report of the DoH Expert Advisory Committee on Biocides (Department of Health, 1989). The planktonic phase of the cooling tower system was heavily colonised with *L. pneumophila* at the end of operational Mode 1 (Figure 3.6) and biofilm samples indicated that *L. pneumophila* had also colonised the surfaces within the system (Table 3.11). After chlorination at 50 ppm free available chlorine was carried out no legionellae or HPC bacteria were recovered from water samples collected from either the purge valve or the low point drain of the cooling tower system (Table 3.16). Moreover, legionellae were not isolated from biofilm slides collected after hyperchlorination.

Legionellae were not isolated from swabs of the pack and sediment at the bottom of the pond, however *L. pneumophila* was isolated from a swab collected from the sediment at

the bottom of the warm-side of the heat exchanger. Heterotrophic plate count bacteria, 8 x 10 CFU l^{-1} were also isolated from the swab collected from this site, approximately 50% of these were identified as *Pseudomonas stutzeri* and the remainder an *Aeromonas* species. The number of *L. pneumophila* in the heat exchanger sediment was determined to be 1 x 10^5 CFU m^{-2}. The concentration of HPC bacteria in the small volume of residual water which remained in the heat exchanger after chlorination and drain down was 7.65 x 10^5 CFU l^{-1}. Around 70% of the heterotrophs isolated in this sample were identified as being *P. stutzeri* the majority of the remainder were typed as an *Aeromonas* species. No legionellae were isolated from this water. The mean water temperature and pH during this trial were 17.5°C and 9, respectively.

Hyperchlorination of the cooling tower system was carried out at the end of Mode R2 following the same protocol as above. No legionellae were present in either the planktonic or biofilm samples collected before or after this disinfection. The heterotroph population although still detectable was reduced by 1000-fold in the bulk water. Biofilm populations of heterotrophs were also reduced by at least 1000-fold (Table 3.17). The mean water temperature and pH were 22.6°C and 8.7, respectively. Bacteria isolated from a biofilm slide retrieved in the pond after chlorination were identified as *Pseudomonas vesicularis*, a *Pseudomonas* species and an *Aeromonas* species, the latter was also isolated from the post chlorination water sample.

Table 3.14 Significant correlations ($P < 0.05$) between numbers of *Legionella pneumophila* and water chemistry parameters in the cooling tower.

Parameter	r[a]	Probability[b]
Hardness ($CaCO_3$ equivalent)	-0.4729	0.000
Calcium	-0.4237	0.002
Potassium	-0.3411	0.009
Zinc	-0.3193	0.014
Magnesium	-0.2422	0.050

[a] Product-moment correlation coefficient.

[b] Probability of occurring by chance.

Table 3.15 Significant correlations ($P < 0.05$) between numbers of heterotrophic plate count bacteria and water chemistry parameters in the cooling tower.

Parameter	r[a]	Probability[b]
Silicate	0.7725	0.000
Chloride	0.5658	0.000
Hardness ($CaCO_3$ equivalent)	0.5122	0.001
Calcium	0.5019	0.002
Potassium	0.3211	0.039
Zinc	-0.4808	0.003

[a] Product-moment correlation coefficient.

[b] Probability of occurring by chance.

Table 3.16 Effect of hyperchlorination (50 ppm for 4 hours) on concentrations of planktonic *Legionella pneumophila* and heterotrophic bacteria in the cooling tower

Sample	Bacterial count (CFU l^{-1})	
	Before hyperchlorination	After hyperchlorination
L. pneumophila	4.65×10^5	BDL[a]
Heterotrophs	4.50×10^8	BDL

[a] BDL = below detection level of 100 CFU l^{-1}.

Table 3.17 Effect of hyperchlorination (50 ppm for 4 hours) on sessile and planktonic populations of heterotrophic bacteria in the cooling tower.

Sample	Heterotroph count (CFU m^{-2})	
	Before hyperchlorination	After hyperchlorination
Pond biofilm	1.067×10^9	7×10^5
Heat exchanger biofilm	1.083×10^9	BDL[a]
Below pack biofilm	4.708×10^8	BDL
Above pack biofilm	1.530×10^8	BDL
Drift eliminator biofilm	BDL	BDL
Water (CFU l^{-1})[b]	1.54×10^9	1×10^6

[a] BDL = below detection level of 1.667×10^5 CFU m^{-2}.

[b] Change in units to CFU l^{-1} for the planktonic samples.

CHAPTER 4: DISCUSSION

4.1 CONTINUOUS CULTURE PHYSIOLOGY EXPERIMENTS

4.1.1 Overview of physiology in chemostat culture

The use of a chemically defined medium in conjunction with continuous culture has permitted a detailed investigation of how the growth environment modulates the physiology and morphology of *L. pneumophila*. Serine provided the principal source of carbon, nitrogen and energy. Only 15 mM ammonia was produced from the 18.5 mM serine metabolised at 24°C, so the remainder was presumably incorporated into biomass, since the deamination of serine should result in a stoichiometric production of ammonia.

The production of a soluble brown pigment is common to a number of *Legionella* species (Vessey *et al.*, 1988) and has been reported in the stationary phase of batch cultures of *L. pneumophila* (Pine *et al.*, 1979; Warren & Miller, 1979; Ristroph *et al.*, 1981). It was suggested that pigment production appears to be typical of a secondary metabolite only appearing as the cultures enter stationary phase (Warren & Miller, 1979). However, Berg *et al.* (1985) found pigment formation in chemostat cultures of *L. pneumophila* using a complex medium where the culture, by definition, would be in the exponential phase. Pigment production is influenced by the concentration of tyrosine in the medium (Baine *et al.*, 1978; Warren & Miller, 1979), and it is likely that pigment formation results when there is an excess of this amino acid. In the current study pigmentation was not observed during growth of chemostat cultures of *L. pneumophila* where tyrosine was the growth limiting nutrient, except when the medium was supplemented with tyrosine. Berg *et al.* (1985) used a yeast extract medium in their chemostat and so it is possible that at low dilution rates the cultures were not tyrosine limited.

The biomass concentration of cultures of Corby, the isolate associated with an outbreak, was reduced by 55% when grown at 24°C rather than 37°C; in contrast strain 74/81, which was isolated from environmental source not associated with an outbreak, was not so critically affected by low growth temperature, the biomass concentration was only reduced by 10% when the culture temperature was lowered from 37°C to 24°C. This may be indicative of different temperature optima for outbreak and non-outbreak strains.

4.1.2 Morphology

This study, in common with others has shown that temperature has a pronounced effect on the cellular morphology of *L. pneumophila*. Pine *et al.* (1979) used a similar medium supplemented with casein hydrolysate and found coccobacillary *L. pneumophila* at 25°C which became pleomorphic above 30°C. Later, Berg *et al.* (1985) observed pleomorphism at 37°C and 44°C in continuous culture using a complex yeast extract medium supplemented with starch and cysteine. However, neither study commented on the presence or absence of flagella. The observation in the current study that flagella were produced at 24°C and not at higher temperatures is consistent with earlier reports. Dennis (1986) noted induction of flagella in continuous culture at low temperature, using a complex yeast extract medium, and Ott *et al.* (1991) showed that in two strains of *L. pneumophila* the proportion of cells bearing flagella decreased with increasing growth temperature. Temperature dependent expression of flagella has been reported in a number of species of bacteria with expression at low, but not high temperature. These include *Yersinia enterocolitica* and *Y. pseudotuberculosis* (Cornelis, 1992), *Listeria monocytogenes* (Peel *et al.*, 1988), *Campylobacter jejuni* (Aguero-Rosenfeld *et al.*, 1990). The motility provided by flagella confers obvious benefits on aquatic microorganisms for survival in the environment. However, the possession of flagella by legionellae *in vivo* may not only be unnecessary for virulence, as might be suggested by similar expression of flagella in virulent and avirulent variants of *L. pneumophila* Philadelphia 1 (Ott *et al.*, 1991), but may indeed

have a detrimental effect on survival of the bacteria *in vivo* due to their antigenicity. Therefore the absence of flagella in *L. pneumophila* grown at 37°C may fortuitously enhance survival *in vivo* and consequently pathogenicity.

Extracellular protease production was also influenced by growth temperature with greater activity evident in the supernatants of cultures grown at 37°C, however protease constituted a larger portion of the extracellular protein production at 24°C. Protease appeared to be preferentially produced with respect to total extracellular proteins during sub-optimal growth conditions *i.e.* low temperature or iron-limited. *Legionella pneumophila* is asaccharolytic and protease production may represent an important means of obtaining assimilable carbon and nitrogen. The significance of protease in the pathogenesis of legionellosis is unclear with a report which suggest that it is not required for intracellular growth or cell killing (Szeto & Shuman, 1990) conflicting with its ability to cause lesions similar to those seen in Legionnaires' disease (Baskerville *et al.*, 1986).

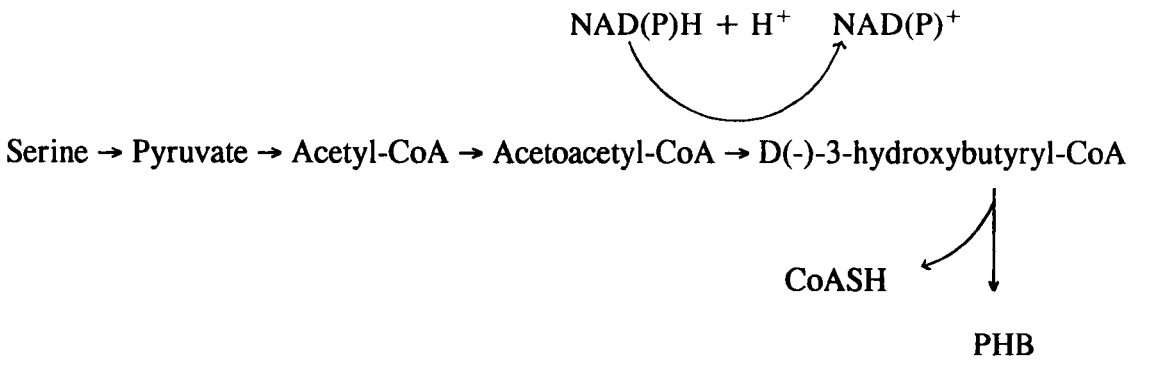
Although there are numerous examples of growth environment influencing cell morphology (*e.g.* Herbert, 1961; Brown & Hough, 1965) it should be noted that agitator speed can have an influence on the mean cell volume of chemostat cultures (Wase *et al.*, 1982). As dissolved oxygen concentration in the chemostat was maintained by varying agitator speed and given that increased oxygen demand would result from greater biomass, coupled with lower oxygen solubility at the higher temperatures, these conditions would result in different agitator speeds according to the growth temperature. Therefore, the possibility that variations in agitator speed could influence cell size should not be ignored.

4.1.3 Accumulation of intracellular storage compound

Intracellular granules were discernible in electron micrographs and were observed by light microscopy after staining with the lipid stain Sudan Black, or as refractile

inclusions when viewed with a DIC microscope. Although the presence of intracellular granules has not been commented on previously by physiologists and biochemists working with legionellae, such granules have been observed *in vivo* by microscopists. For example, Chandler *et al.* (1979) noted the presence of lipid granules in legionellae growing in alveolar macrophages at 37°C. Similar observations were made for legionellae growing in yolk sac membranes (Rodgers, 1979), the protozoan *Tetrahymena pyriformis* (Fields *et al.*, 1986) and the amoeba *Acanthamoeba palestensis* at 35°C (Anand *et al.*, 1983). By contrast, few granules were observed in legionellae growing in *Acanthamoeba polyphaga* at a lower temperature of 30°C (Rowbotham, 1984). The apparent influence of temperature on granule formation was confirmed by the present study, in which maximum granule formation was noted at 30°C, significantly fewer being observed at 37°C and very few at 24°C. Since the abundance of granules did not correlate to the PHB content of the biomass, and the granules do not appear to be polysaccharide, their precise nature remains to be established. However chemical analysis did confirm that the cells contained the storage compound PHB. This polymer comprised 4.3% of the cell dry weight when bacteria were grown at 24°C and decreased to 1.5% when the growth temperature was increased to 37°C. Synthesis of PHB was not completely abolished, probably because the bacteria were not carbon-limited, as serine remained in excess at all temperatures. At 24°C *L. pneumophila* exhibited the lowest efficiency of biomass production for serine metabolised (*i.e.* Y_{serine}), however, the PHB content of the cells was maximal at this temperature suggesting that growth is uncoupled and the accumulation of this storage compound is favoured at lower temperatures. An important adjunct to this work would be to establish if iron-limitation resulted in increased PHB accumulation as might be expected due to uncoupled metabolism of serine. However, time did not permit this further investigation.

The assumed pathway for PHB synthesis from amino acids such as serine is via pyruvate and acetyl CoA (Dawes & Senior, 1973):



The synthesis of a carbon reserve such as PHB would provide an essential source of carbon and energy for survival in low nutrient environments. Legionellae are able to metabolise exogenous ß-hydroxybutyric acid as a carbon source (Section 3.4.1 and Mauchline & Keevil, 1991) suggesting that they are able to grow following hydrolysis of the intracellular PHB reserve. This would explain their ability to maintain their ATP content for many months with no additional nutrient source (West *et al.*, 1989). It is also possible that PHB provides a carbon and energy supply for growth in macrophages and amoebae. Indirect evidence has been reported for the accumulation of PHB by a number of *Legionella* species (Helm *et al.*, 1991; Watt *et al.*, 1991), suggesting that PHB accumulation and use may be a common strategy amongst legionellae. Furthermore, PHB may be useful as a chemical marker of legionellae in environmental samples (Helm *et al.*, 1991; Watt *et al.*, 1991).

The PHB biosynthetic pathway also provides a mechanism to re-oxidise NAD(P)H to NAD(P) to continue essential metabolic processes. Aerobes normally oxidise NAD(P)H via the respiratory chain with oxygen as the terminal electron acceptor. Legionellae, however, behave as microaerophiles (Locksley *et al.*, 1982; Hoffman *et al.*, 1983) and were inhibited by oxygen concentrations above 1 mg l^{-1} under the conditions in this

study. They may therefore need alternative electron acceptors to recycle cofactors. PHB synthesis serves as an electron sink for reducing power which accumulates as a consequence of oxygen limitation; the flow of electrons along the electron transport chain to oxygen is impeded and thus they can be diverted to the reductive step in polymer synthesis (Senior & Dawes, 1973).

4.1.4 Temperature effects on fatty acid profile and membrane fluidity

Wait (1988) reported that there was no significant difference between the fatty acid profiles of *L. pneumophila* grown on BCYE agar or in continuous culture using complex yeast extract medium at comparable temperatures, except that the production of 9,10 methylene hexadecanoic acid (cyclopropane 17) was much reduced in the chemostat. Similar results were obtained in the present study using a defined medium, except that at 24°C the cyclopropane 17 acid is completely absent. In other microorganisms (Halper & Norton, 1975), including *L. feeleii* (Moss *et al.*, 1983), it has been observed that cyclopropane acids accumulate as the culture ages, the rate of their synthesis being lowest in log-phase. Hence it is not surprising that concentrations are low in cells grown in continuous culture, where the majority of cells are by definition in log-phase.

Bacteria can only grow if their cell membranes are in a largely fluid state; *i.e.* if the temperature is above the gel to liquid-crystalline transition temperature of their membrane lipids so as to ensure the maintenance of essential transport functions. Thermotolerant organisms, such as legionellae, thus require adaptive mechanisms to ensure that their membranes are fluid across a wide temperature range. Generally, such adaptive strategies involve the synthesis of membrane constituents of lower melting point at reduced growth temperature. This can be achieved by switching fatty acids from saturated to unsaturated, from unbranched to branched, from iso- to ante-iso branched, by reducing acyl chain length, and by alteration of phospholipid type (Melchoir, 1982). The present data suggest that in *L. pneumophila* the major

mechanism at work is increased production of unsaturated fatty acyl chains at low growth temperature. There is no evidence of any tendency towards acyl chain shortening. It is, however, significant that the reduction in saturated acid content is achieved almost entirely at the expense of straight chain and iso-branched acids, which have a significantly higher melting temperature than their ante-iso branched isomers (Rilfors *et al.*, 1978). The alterations in phospholipid composition are less easy to interpret. The reduced content of phosphatidylcholines at low temperature seems surprising, as phosphatidylcholines have a 20°C lower melting temperature than phosphatidylethanolamines with identical acyl substituents (Chapman *et al.*, 1967; Wilkinson & Nagle, 1981). However, since this is due to packing effects and the greater ability of the ethanolamine head group to form inter-molecular hydrogen bonds, the effect is likely to be less pronounced in mixed phospholipids. It would seem likely that increasing the proportion of unsaturated substituents in phospholipids of all types has a greater effect on phase transition temperatures than alterations to the polar head group.

4.2 MODIFIED BIOLOG SYSTEM FOR METABOLIC PROFILING AND IDENTIFICATION OF LEGIONELLAE

4.2.1 Metabolic profiling of legionellae

The *Legionellaceae* have been considered to be fastidious due to their inability to metabolise carbohydrates or grow on a range of routine laboratory media. The present study showed, as was expected, that the legionellae investigated were most metabolically active towards amino acids and their derivatives in the BIOLOG panel of substrates which agrees with previous work on the metabolism of this genus (Pine *et al.*, 1979; Muller, 1981; Tesh *et al.*, 1983; Franzus *et al.*, 1984; Keen & Hoffman, 1984), and were, in addition, able to metabolise a range of carboxylic acids and their derivatives, including acetate, propionate, succinate, monomethyl succinate and methylpyruvate. The results of the current investigation agreed with those of Tesh et

al. (1983) who found that addition of pyruvate, succinate and acetate stimulated oxygen uptake by suspensions of washed cells of *L. pneumophila*, but that α-ketoglutarate did not. Although cell-free extracts of *L. pneumophila* have α-ketoglutarate dehydrogenase activity (Hoffman & Pine, 1982; Tesh *et al.*, 1983; Keen & Hoffman, 1984), whole cells fail to oxidise this substrate to any appreciable level (Tesh *et al.*, 1983; Keen & Hoffman, 1984). This led Tesh et al. (1983) to postulate that *L. pneumophila* was unable to transport this compound. The results of the present study support this: no metabolism of α-ketoglutarate was found in any of the strains examined, although the other keto-acids in the panel were metabolised by almost all of the strains investigated. All the strains tested metabolised methylpyruvate, ß-hydroxybutyrate, and most metabolised Tween 40 and Tween 80 suggesting that they possesses esterase activity, which is consistent with previous reports (Muller, 1981; Nolte *et al.*, 1982; Vessey *et al.*, 1988). However, this may not be due to specific esterase activity but may be a consequence of protease activity. Specific aminopeptidase activities for *L. pneumophila* have been reported by Muller (1981), while in this study it was found that several dipeptides were metabolised by legionellae. In agreement with the observations of Weiss and Westfall (1984) gamma-aminobutyrate was catabolised by a number of strains of *L. pneumophila*.

The results of the BIOLOG study indicate that alanine, asparagine, aspartate, glutamate, leucine, proline, serine and threonine are catabolised by *L. pneumophila* and a number of the other *Legionella* species tested which is consistent with earlier studies (Pine *et al.*, 1979; George *et al.*, 1980; Ristroph *et al.*, 1981; Tesh & Miller, 1981; Keen & Hoffman, 1984). This is also consistent with the utilisation of these amino acids by chemostat cultures of *L. pneumophila* described earlier (Section 3.1.1 and Table 3.1) The lack of activity towards histidine is surprising given that this amino acid was utilised by both strains of *L. pneumophila* in the chemostat experiments described earlier. Franzus *et al.* (1984) reported that histidine was metabolised to give an acid product, this would be consistent with production of urocanate, the first intermediate in

the conversion of histidine to glutamate. Subsequently glutamate is converted to α-ketoglutarate by glutamate-aspartate transaminase and thus enters the Krebs cycle (Keen & Hoffman, 1984). All the strains of *L. pneumophila* and five of the other *Legionella* species metabolised urocanate, but all (with the exception of *L. pneumophila* sergroup 5) failed to catabolise histidine which is consistent with the lack of histidase activity reported by Keen and Hoffman (1984). This suggests the possibility that exogenously available histidine is preferentially used for biosynthesis by legionellae, but urocanate is directed towards energy yielding catabolism. Likewise, phenylalanine was not oxidised in the BIOLOG study, however it was utilised by chemostat cultures of *L. pneumophila* which suggests a biosynthetic role for this amino acid.

Since all the strains investigated in this study metabolised ß-hydroxybutyrate, the monomer of PHB, this supports the notion that PHB may play a significant role as a carbon and energy storage compound for survival in oligotrophic environments. In addition, this study provides further evidence of the profound effect of dissolved oxygen concentration on legionellae. Increased cell density of inocula overcame the toxic effects of oxygen and allowed rapid metabolism of substrates, which is similar to the observations of Jannasch (1977) pertaining to microaerophilic spirillia, and is consistent with experience of low inoculum concentrations in the chemostat requiring to be gassed with nitrogen to prevent wash-out.

The metabolic profiling of legionellae, and *L. pneumophila* in particular, by the modified BIOLOG system, proved a useful adjunct to the continuous culture physiology study described previously. As well as supporting observations on amino acid utilisation by *L. pneumophila*, the BIOLOG experiments provided evidence for the hypothesis that *L. pneumophila* uses PHB as a carbon and energy reserve.

4.2.2 Biochemical identification of legionellae

Identification of legionellae at the species level is difficult; indeed some species can

only be identified by DNA hybridisation studies. The practice of defining *Legionella* species on the basis of DNA/DNA hybridisation has resulted in the creation of genospecies with the consequence that *L. anisa, L. bozemanii* and some other blue-white autofluorescent species can only be delineated on the basis of DNA analysis (Harrison & Saunders, 1994). A similar situation exists with the red autofluorescent species, *L. erythra* and *L. rubrilucens*, which cannot be reliably distinguished using biochemical, chemotaxonomic or serological tests (Saunders *et al.*, 1992). Speciation is usually achieved by a few phenotypic characteristics and tests combined with antigenic analysis using unabsorbed rabbit hyperimmune antisera. However, this practice has drawbacks. Many species and serogroups have common antigens, leading to cross-reactions. This can be overcome by cross-absorption to produce monospecific antisera. However, this presents other difficulties: it is labour intensive; the description of new species requires reevaluation and possibly additional absorption procedures; antisera are evaluated against a few representative strains of each species, but antigenic variation may be large; and such antisera are not commercially available.

Several phenotypic tests are useful in identifying legionellae to the genus level: these are the requirement for L-cysteine, colony morphology, Gram stain characteristics, catalase and oxidase reactions and the absence of nitrate reduction. Others such as autofluorescence, hippurate hydrolysis, pigment production and gelatinase production are useful in speciating legionellae (Harrison & Taylor, 1988).

The results described previously in Section 3.4 suggest that when employing this modified procedure, the BIOLOG bacterial identification system has the ability to identify legionellae at least to species level and that both its specificity and sensitivity are good. In order to create stable databases it will be necessary to characterise multiple strains from all known species. Although the database produced in this study was derived from reaction data for a relatively small number of previously identified type

strains, it has demonstrated that it is possible to identify legionellae by phenotypic means. The system correctly identified non-type strains isolated from environmental and clinical sources. The method described in this thesis offers several advantages over other the phenotypic schemes developed previously (Vessey *et al.*, 1988; Fox & Brown, 1989), in that more tests contribute to the resolution of species and, of practical significance, the modified BIOLOG system offers a simpler, quicker and more easily standardised procedure which only requires single culture, suspension and inoculation steps. The utility of this system based on substrate utilisation is in contrast to an earlier study which concluded that a practical diagnostic test for *Legionella* species based on amino acid utilisation was not achievable (Franzus *et al.*, 1984). The current work demonstrates that the inclusion of carboxylic acids, esters and polymers in addition to amino acids increases the resolving power of a substrate utilisation based identification system when applied to typing *Legionella* species.

Slightly more substrates were utilised when the BIOLOG plates were inoculated with cultures grown on BCYE for 24 hours: use of a shorter incubation time for the inoculum may be advisable when the system is being used to investigate the physiology of strains. In the interest of standardisation and simplicity, however, inocula grown for 72 hours seem suitable when using the system to identify isolates. Incubating the test plates in a low oxygen atmosphere or at high cell density allows much more rapid development of positive tests, thus facilitating identification after only 24 hours incubation. To increase the ability of the system to differentiate between *Legionella* species it would be desirable to increase the number of substrates which can be potentially used by a *Legionella* isolate. Thus the system's resolving power for the *Legionellaceae* could be enhanced by the replacement of the carbohydrate substrates (legionellae are essentially asaccharolytic) by substrates that can be utilised by these bacteria.

Phenotypic identification of putative *Legionella* isolates by the method described above

would be a useful tool for routine laboratories. The relatively modest expense of purchasing the software and standard BIOLOG plates would be offset by reduction in labour costs. By utilising the modified BIOLOG system, which provides a simple, rapid and standardised procedure, it is now possible to address the points that Brenner (1986) made in his review of the classification of the *Legionellaceae*: "Representative numbers of strains from all species must be systematically studied by all the phenotypic methods available . . . to determine whether a means of phenotypically speciating legionellae is achievable" and if so to "decide if there are valid phenotypic grounds for more than one genus in the family *Legionellaceae*".

4.3 INFLUENCE OF GROWTH TEMPERATURE ON VIRULENCE

This study clearly demonstrates that growth temperature has a profound effect on the virulence of *L. pneumophila* with bacteria grown at 37°C being significantly more virulent than when grown at 24°C. Cultures of *L. pneumophila* grown at 24°C were avirulent; however a virulent phenotype was re-established when the temperature was raised to 37°C. The latter observation gives a strong indication that the event which occurred was a reversible modulation of virulence rather than the selection of a mutant or clonal sub-population. If the lower culture temperature favoured a mutant or sub-population sufficiently, the new strain would establish itself and completely displace the native organisms (Powell, 1958). If this were the case the original population would not be able to re-establish itself when the temperature permissive for virulence was restored. The virulence modulation reported here is clearly distinct from the phenomenon reported by McDade and Shepard (1979) where *L. pneumophila* becomes avirulent after passage on supplemented Mueller-Hinton agar, and which Catrenich and Johnson (1988) later concluded to be a one-way phenomenon.

The possibility that the regulation of *L. pneumophila* virulence is based on temperature difference between the environment and that of a human host, has a

parallel in *Shigella* species where the bacteria are invasive when cultivated at 37°C but are not invasive when grown at 30°C (Maurelli *et al.*, 1984). Other studies which have investigated the effect of temperature on the virulence of *L. pneumophila* have yielded differing results: Berg *et al.* (1985) found no difference in the pathogenicity of *L. pneumophila* when grown at 37°C or 44°C in continuous culture using a complex medium; Edelstein *et al.* (1987) found that *L. pneumophila* was more virulent when grown at 25°C than when grown at 41°C in buffered yeast extract broth in batch culture. The authors of the last study commented that their findings were contrary to what they had expected and offered several reasons for this. The reason which they considered most likely was that growth in their culture system at 25°C was more representative of the restricted conditions found in the environment where the doubling times are likely to be longer than those obtained at 41°C. The possibility that doubling time has a role in determining virulence was eliminated in this present investigation since all the cultures had the same specific growth rate irrespective of growth temperature. It should also be noted that the routes of infection in all of these studies were different; only in the present study was inoculation achieved by the natural route of infection, by inhalation.

The temperatures in man-made water systems vary considerably but can often be conducive to growth of legionellae: hot water systems in which the storage tanks were maintained at between 43°C and 45°C have been shown to be colonised with *L. pneumophila* (Plouffe *et al.*, 1983), and more recently a hot water system operated at 43°C has been implicated in an outbreak of Legionnaires' disease (Colville *et al.*, 1993). Water temperatures found in cooling towers have been reported in a number of studies: 8.3 to 35.2°C with maximum legionellae numbers at 26.3 to 29.9°C (Yamamoto *et al.*, 1992); 19 to 40°C (Kusnetsov *et al.*, 1993); 13 to 35°C (Howland & Pope, 1983). It is worth remembering that the water temperature is most commonly measured in the pond which is the coolest part of the system, however the temperature at the condenser of the refrigeration unit (in the case of air conditioning applications) or

at the process requiring cooling will inevitably be higher. The condensing temperature of the majority of water cooled condensing units lies between 35°C and 45°C (Hill *et al.*, 1990). Since the opportunity for *L. pneumophila* to experience growth temperatures in the region of 37°C in such environments clearly exists it is conceivable, in the light of the findings reported in this thesis, that growth of the bacterium in such water systems may result in the induction of a virulent phenotype. In this way hot water systems, evaporative condensers and cooling towers may not only act as amplifiers and disseminators of legionellae, but may also prime the organism for causing disease in man. This conclusion adds additional weight to the guidance provided for the safe operation of domestic water systems (HSE, 1991; NHS Estates, 1993), *i.e.* that services should not be operated in the temperature range likely to lead to growth of legionellae. Furthermore this work should suggest a codicil: that particular attention should be given to avoid conditions which would allow growth in the region of 37°C.

Two of the conclusions of this present study, that growth at 37°C and the availability of iron increase the virulence of *L. pneumophila*, appear to support the observations of Christensen *et al.* (1984), that *L. pneumophila* was more infective to guinea pigs when it was isolated from closed-cycle rather than once-through cooling systems. Since the concentrations of viable *L. pneumophila* in the infective and non-infective samples were similar it suggests that some other mechanism was at work other than increased densities of legionellae resulting in increased likelihood of infection. *Legionella pneumophila* in closed-cycle or recirculating cooling systems will experience growth at elevated temperatures for an extended period of time and because of the concentation effect of such systems the levels of nutrients, including iron, will be increased. Bacteria in once through systems will only be subject to one brief passage at elevated temperature and the concentrations of nutrients will be the same as in the source water in the condenser, with a slight increase after evaporation, *i.e.* in the cooling tower pond.

4.4 IRON LIMITED GROWTH

This study demonstrated for the first time that *L. pneumophila* could be grown in chemostat culture under conditions of iron-limitation and that around 0.3 μM of iron was sufficient to support a biomass of 0.583 g (dry weight) l^{-1} . These findings are in agreement with earlier reports of the concentration of iron required to support growth of *L. pneumophila*: Warren and Miller (1979) found that chemically defined medium with an iron concentration of less than 1.5 μM (the limit of their detection system) would support the growth of *L. pneumophila* (Knoxville) with only slight depression of the cell yield; Goldoni *et al.* (1991) reported that around 0.9 μM iron was sufficient to support growth of *L. pneumophila* in static and aerated batch cultures and that growth was enhanced in the former; and Reeves *et al.* (1981) observed that the concentrations of metals required for optimal growth (including iron) varied between strains, but that 0.5 μM iron could support growth of all six strains to some extent. These observations are in keeping with the iron requirement of 0.3 to 1.8 μM usually associated with Gram-negative bacteria (Weinberg, 1974). The observation that 2.8 μM iron was sufficient to support maximal biomass was consistent with the report of Johnson *et al.* (1991) that virulent *L. pneumophila* cells require 3 μM iron. Cultures grown with less iron (*i.e.* iron-limited) were rendered avirulent.

The observation that iron-limited cultures consist of short rods, unlike iron-replete cultures which are pleomorphic, comprising both short and long forms, is in broad agreement with earlier observations (Ristroph *et al.*, 1981). These observations are contrary to reports of the production of filamentous forms during iron-limited growth in a number of bacterial species (Light & Clegg, 1974). The increase in Y_{iron} and associated reduction in Y_{serine} for *L. pneumophila* under iron-limitation is comparable to effect of iron-limitation on these yield coefficients for *Torulopsis utilis* grown iron-limited in continuous culture (Light & Clegg, 1974). Clearly *L. pneumophila* in common with other bacteria increases it efficency of iron utilisation under limiting conditions. Subsequent experiments using the culture system described above indicated

that there was a 54% decrease in the iron content of the biomass under iron-limitation (James *et al.*, 1995).

4.4.1 Influence of iron-limitation on virulence

Iron-limited growth was found to modulate the virulence of *L. pneumophila*, with cells grown iron-limited being less virulent than those grown under iron-replete conditions. This reduction in virulence, like the temperature modulation described earlier, was reversible with the virulent phenotype being restored on returning the culture to iron-replete conditions. The finding that *L. pneumophila* was less virulent after iron-limited growth is counter-intuitive since iron-restriction is known to enhance the virulence of a number of pathogens (Pappenheimer & Johnson, 1936; Pappenheimer & Shaskan, 1944; Meuller & Miller, 1945; Bjorn *et al.*, 1978; Brener *et al.*, 1981; Ombaka *et al.*, 1983; Brown *et al.*, 1984; Keevil *et al.*, 1989). However, legionellae have most probably evolved as environmental organisms colonising a wide variety of aqueous environments and growing intracellularly in amoebae and protozoa. Legionellae are only opportunistic pathogens of man. As such it is not unreasonable to find that the response of *L. pneumophila* to iron-limitation differs from that of bacteria which have evolved as human pathogens. A possible explanation of the reduction in virulence described above may be the physiological state of iron-limited *L. pneumophila* prior to infection of the guinea-pigs.

Legionella pneumophila has 7 iron-containing proteins, including an aconitase and an iron superoxide dismutase (FeSOD; Mengaud & Horwitz, 1993). The major iron-containing protein (MICP) is an aconitase, which contains 4 atoms of iron per molecule and it has been estimated that each cell contains around 10^5 molecules of MICP, accounting for a significant requirement for iron (Mengaud & Horwitz, 1993). Aconitase is an enzyme [citrate (isocitrate) hydrolyase] containing a Fe-S cluster and catalysing the stereospecific dehydration-rehydration of citrate to isocitrate in the Krebs cycle. The active form contains a [4Fe-4S] cluster. Under oxidative conditions it may

lose one iron atom to give [3Fe-4S], which lacks enzymatic activity. Since the Krebs cycle is the main route of energy production and carbon assimilation in *L. pneumophila* (Hoffman & Pine, 1982), aconitase activity is required for the growth of this organism. Furthermore, MICP may fulfill other essential functions in *L. pneumophila* such as: iron storage; regulation of gene expression in response to cellular iron; or as a defence against toxic oxygen or nitrogen intermediates (Mengaud & Horwitz, 1993).

Legionella pneumophila contains 2 superoxide dismutases, a cytoplasmic iron enzyme (FeSOD) and a periplasmic copper-zinc superoxide dismutase (CuZnSOD; Sadosky *et al.*, 1994). *Legionella pneumophila* is sensitive to toxic oxygen metabolites (Locksley *et al.*, 1982; Lochner *et al.*, 1983; Jepras & Fitzgeorge, 1986). Payne and Horwitz (1987) suggested that due to its route of entry into phagocytes, *L. pneumophila* does not trigger the respiratory burst; however, superoxide production has been detected in human monocytes in response to infection with *L. pneumophila* (Summersgill *et al.*, 1990). More recently, Sadosky *et al.* (1994) demonstrated that FeSOD is essential for viability of *L. pneumophila* and that the CuZnSOD cannot compensate for its absence. Although the gene coding for FeSOD, *sodB*, is constitutively expressed, the amount of active enzyme could be influenced by the availability of iron, since it is required as cofactor to convert the apoenzyme to the active holoenzyme.

Given that the iron content of *L. pneumophila* is reduced by around 54% when it is grown iron-limited (James *et al.*, 1995), it is conceivable that the reduction in the concentration of one or more of these proteins in response to iron-limitation prior to challenge, may account for the decrease in virulence reported in this study. Further studies are necessary to investigate the molecular basis for this reduction of virulence.

4.4.2 Siderophore activity

As stated in the Section 1.6.2 there is some disagreement about how legionellae satisfy their requirement for iron. Neither hydroxymate nor phenolate siderophores have been

detected in supernatants of *Legionella* species during iron-restriction (Reeves *et al.*, 1983; Goldoni *et al.*, 1991). Siderophore activity, mean value of 10.32 μM (desferrioxamine methanesulphonate; DFOM equivalent), was detected in the supernatants of *L. pneumophila* cultures grown iron-limited in this present study using an universal siderophore assay which detects iron sequestering activity rather than chemically detecting the functional groups associated with particular classes of siderophores (Schwyn & Neilands, 1987). No siderophore was detected in iron-replete cultures under the same defined culture conditions. Utilising the same assay Goldoni *et al.* (1991) also detected siderophore activity in supernatants of cultures of *L. pneumophila, L. bozemanii, L. dumoffii, L. jordansis, L. micdadei* and *L. oakridgensis*. They found 20 μM and 6 μM siderophore activity (expressed as DFOM equivalent) in static and aerated iron-restricted batch cultures, respectively, after 10 days growth but none in iron-replete cultures of *L. pneumophila*. From the results presented in this thesis and the work of Goldoni *et al.* (1991) it is clear that *L. pneumophila* expresses some kind of siderophore which falls into the third category of "miscellaneous" siderophores described by Neilands (1984) as being neither hydroxamate nor catechol siderophores but probably amino or imino carboxylic acids. The reaction kinetics of the assay suggests that the *L. pneumophila* siderophore may be a polyamino carboxylic acid. Only biological assays such as the one used by Warren and Miller (1980) and the assay used in this present investigation are able to detect presence of such siderophores. Elaboration of a siderophore would have obvious survival advantages for *L. pneumophila*. The availability of iron *in vivo* is restricted in both the extracellular (Muller *et al.*, 1983) and intracellular environments (Byrd & Horwitz, 1989) in response to infection. The availability of iron in water systems may also be severely restricted as the more soluble ferrous iron is readily oxidised to insoluble ferric hydroxides and oxyhydroxides (Griffiths, 1987).

Production of siderophore activity and an increase in the efficiency of iron metabolism during iron-limitation suggest that *L. pneumophila*, in common with other

microorganisms, possess a complex regulatory network which controls metabolism so as to utilise the available iron efficiently and maintain essential cellular functions. In a number of bacterial pathogens, the regulation of gene expression by iron is mediated by the Fur (ferric uptake regulation) repressor protein, which is coded for by the *fur* gene (Hantke, 1984; Litwin *et al.*, 1992; Prince *et al.*, 1993). The recent report that *L. pneumophila* and other *Legionella* species possess *fur* genes and that *L. pneumophila* Fur exhibits 72% similarity with Fur proteins of other bacteria (Hickey & Cianciotto, 1994), combined with the results of the current study, suggest that *L. pneumophila* alters the expression of its iron uptake system in response to the concentration of iron. This would suggest that *L. pneumophila* employs similar genetic control of siderophore production to other bacterial pathogens. In other bacteria Fur also regulates the expression of other potential virulence factors such as exotoxins and haemolysin (Litwin & Calderwood, 1993; Prince *et al.*, 1993). The present investigation demonstrated that iron-limited growth resulted in a four-fold reduction in protease production (determined as azocaseinase activity) by *L. pneumophila* compared to iron-replete conditions. *Legionella pneumophila* protease, however, is not required for intracellular growth or cell killing (Szeto & Shuman, 1990).

Although iron-limited growth resulted in production of siderophore-like activity by cultures of *L. pneumophila* this did not correspond with enhanced virulence; potential reasons for this are discussed above. One conclusion from these findings is that the iron-scavenging activity of *L. pneumophila* may be of greater importance for survival in the environment than for pathogenicity.

4.5 GROWTH OF *LEGIONELLA PNEUMOPHILA* IN THE MICROBIOLOGICALLY-CONTAINED COOLING TOWER

Considerable effort was expended to mimic the design, construction and operation of commercial evaporative cooling towers as closely as possible, and to simulate the growth environment which legionellae are likely to encounter in such installations. Interviews with cooling tower manufacturers, operators, water treatment specialists and heating and ventilation engineers indicated that this initial aim had been achieved. The density of the inocula (to achieve an initial *L. pneumophila* concentration of around 10^3 CFU l^{-1}) was selected to give a fair representation of the concentation of legionellae which may enter a cooling tower which is receiving its make-up water from a public water supply (Tison & Seidler, 1983; Colbourne & Trew, 1986). The concentration of *L. pneumophila* which was attained in the experimental cooling tower (up to 10^6 CFU l^{-1}) was comparable with legionella densities reported in cooling towers under field conditions (Christopher *et al.*, 1987; Pope & Dziewulski, 1992; Yamamoto *et al.*, 1992; Bentham & Broadbent, 1993; Kusnetsov *et al.*, 1993; Shelton *et al.*, 1994). Legionellae and other bacteria were allowed to colonise the system at their own pace which inevitably resulted in lengthy experiments, but this was viewed as crucial in developing a useful understanding of the sequence and kinetics of colonisation.

A number of technical difficulties had to be overcome to bring this study to fruition. Designing an evaporative cooling tower which could be operated under microbiological-containment proved to be a challenging enterprise. Extensive interdisciplinary background research, imagination and innovation were required to realise the ambition of creating and operating such a novel and unique facility. The reliability and reproducibility of the data obtained was of paramount importance, hence all monitoring equipment was calibrated against traceable standards and standardised protocols were developed for sampling. Problems were encountered in collection of biofilm samples during a portion of the study. The situation was only rectified after

engineering alterations were made to the system. This unfortunately resulted in a shortage of data on surface associated growth for parts of the investigation. The need to overcome difficulties by re-engineering parts of the cooling tower, the duration of the experiments, the constraints imposed by reproducibility and accuracy, all resulted in an inability to carry out all the work planned. However, important data was collected and the ground work laid for further research (discussed in Chapter 5).

This investigation focussed on the influence of operational practices and physicochemical environment on the growth of *L. pneumophila* in the cooling tower. In addition, the size and composition of the populations of heterotrophic bacteria were monitored and possible relationships with concentrations of *L. pneumophila* studied. However, due to time constraints the role of amoebae in the ecology of such systems could not be investigated.

4.5.1 Influence of the data derived from pure culture experiments on the subsequent cooling tower study

The pure culture experiments carried out in defined conditions and discussed in Sections 4.1 and 4.2 provided a basis to understand the influence of the environment on the growth of *L. pneumophila*. Hence the results obtained in these earlier investigations influenced the subsequent cooling tower study. Knowledge of the effect of temperature on the physiology of this bacterium was carried forward from the chemostat experiments to those conducted with the cooling tower and provided a basis for selection of operating temperatures and the rationale behind temperature monitoring. The observation that dissolved oxygen tension had a profound effect on the growth of *L. pneumophila* in chemostat culture and in BIOLOG plates resulted in the decision that DOT should be monitored in the cooling tower.

Although the mean temperatures in the cooling tower were lower than in the experiments conducted at 37°C in the chemostat, the mean daily temperatures in the

heat exchanger were between 24.5 and 31.0°C with the consequence that at least some of the water in the cooling tower was above 24°C, a temperature which supported growth of *L. pneumophila* in the chemostat. The pH of the cooling tower was ≥ 8.5 compared with a controlled value of 6.9 in the chemostat, this was an inevitable consequence of the make-up water. The DOT in the cooling tower fluctuated greatly in response to pump activity but the usual range for this parameter was between 35 and 76% of air saturation, which is considerably higher than in the pure culture experiments. It should be noted that the DOT in biofilms or sediment would be significantly lower and so legionellae may have preferentially colonised these niches.

4.6 COOLING TOWER STUDY: COMPARISON WITH DATA COLLECTED IN FIELD STUDIES

The following section will discuss the results of this study with particular reference to field studies which have investigated factors which may influence the growth of legionellae in evaporative cooling systems. These investigations have been undertaken in different countries and have reported contradictory results (Witherell *et al.*, 1986; Broadbent *et al.*, 1992; Yamamoto *et al.*, 1992; Kusnetsov *et al.*, 1993).

4.6.1 Temperature

In some studies it has been found that concentrations of legionellae correlate positively with pond water temperatures (Broadbent *et al.*, 1992; Yamamoto *et al.*, 1992); in contrast, Witherell *et al.* (1986) and Kusnetsov *et al.*, (1993) reported no correlation between pond water temperatures and legionella colonisation. However, Kusnetsov *et al.* (1993) observed that the mean water temperature in cooling towers containing legionellae was higher than in systems where they was absent. In the present study water temperatures in both the pond and heat exchanger were found to positively correlate with the concentration of legionellae in the water. Mean daily water temperatures during Mode 1 (the mode of operation which resulted in highest legionella

counts) were 23.9°C in the pond and 31.0°C in the heat exchanger, which was in contrast to the temperatures during Mode 2 (the mode of operation which resulted in no legionellae being isolated from the system), where the water temperature in the pond was 21.1°C and 24.5°C in the heat exchanger. This agrees with the findings of Broadbent *et al.* (1992) that legionella concentrations did not increase at pond water temperatures below 16.5°C but rapid multiplication occurred above 23°C. They added the codicil that these temperatures were measured in the coolest part of the system, the pond, and that the water in the condenser and post-condenser pipe-work was typically 5°C warmer, which would suggest that legionellae were not growing below 21.5°C and that rapid multiplication only occurred above 28°C. The only operating regime which resulted in water temperatures above 28°C was Mode 1, all other operating conditions resulted in maximum mean temperatures of below 26°C. This is consistent with the fact that although *L. pneumophila* colonised the cooling tower during Modes 4 and R1 there was no rapid increase in its concentration. These observations are supported by the evidence of field studies in which the likelihood of isolating legionellae increased with increased temperature. These findings are in keeping with the earlier observations that *L. pneumophila* can multiply in water between 25 and 45°C (Wadowsky *et al.*, 1985).

The concentration of heterotrophs in the system appeared to decrease with increased water temperature, it worth considering the possibility that the culture temperature used for determining the total heterotroph count (22°C) may have contributed a bias in favour of isolation of bacteria with lower growth temperature optima.

It should be noted that, in the field studies discussed above, water temperatures were only measured in the cooling tower ponds and that this was only done at the time of sampling (which may have been at intervals of up to one month), whereas in this study water temperatures in the pond (the coolest part of the system) and the heat exchanger (the warmest part of the system) were logged hourly throughout the experiments

whether or not the cooling tower was operating, thus giving a much better indication of the environment that the bacteria were experiencing.

4.6.2 pH

Contradictory results have been reported for the growth of legionellae in relation to pH. No correlation between pH and *L. pneumophila* growth was found in this present study (range of mean pH for modes 8.36 to 8.90) which agreed with the findings of Broadbent *et al.*, (1992; range 7 to 10) and Kusnetsov *et al.* (1993; range 6.1 to 9.3). By contrast, Yamamoto *et al.*, (1992) reported that legionella counts positively correlated with pH (range 7.17 to 9.10) and Witherell *et al.*, (1986) found that the mean pH of systems where *L. pneumophila* was recovered was significantly higher than the mean pH in systems testing negative. However, a negative correlation with pH (range 6.83 to 8.63) has been reported, which led to the suggestion that operating cooling towers at a pH greater than 9 may be useful in controlling the growth of legionellae (States *et al.*, 1987).

4.6.3 Conductivity

A significant negative correlation between conductivity and *L. pneumophila* concentration was found in this study (range 0.6 to 2.6 mS cm^{-1}). Only two of the field studies investigated the influence of conductivity on concentrations of legionellae. Broadbent *et al.* (1992) reported no significant correlation between legionella concentrations and conductivity (range 0.5 to 3.0 mS cm^{-1}). However, Kusnetsov *et al.* (1993) reported that the mean conductivity in cooling towers containing legionellae was 0.39 mS cm^{-1} (range 0.12 to 0.62 mS cm^{-1}), whereas the conductivity in towers from which legionellae were not isolated was 0.52 mS cm^{-1} (range 0.05 to 3.3 mS cm^{-1}).

In the current study the heterotrophic plate count correlated positively with conductivity as might be expected with increasing concentrations of dissolved nutrients.

4.6.4 Dissolved oxygen

No correlation in dissolved oxygen concentration was noted in the present study which agreed with the findings of Kusnetsov *et al.* (1993). Dissolved oxygen concentration was not monitored in the other field studies. It should be noted that it was only possible to monitor the dissolved oxygen tension in the bulk water phase and not in the biofilm phase, where significantly lower oxygen tensions are likely.

The total heterotroph count demonstrated a positive correlation with dissolved oxygen tensions in the system. This was probably due to the increased availability of oxygen enhancing the growth of aerobic species.

4.6.5 Water chemistry

The concentrations of calcium, magnesium, potassium and zinc were all found to correlate negatively with *L. pneumophila* counts in this present study. Only one field study investigated the role of metals in relation to multiplication of legionellae, and it failed to establish significant correlations for any of these ions; potassium concentration was not measured (Kusnetsov *et al.*, 1993). The finding that potassium (range 0.74 to 40.2 ppm) and zinc (range 0.15 to 0.71 ppm) correlated negatively with legionella growth was not consistent with the results of a laboratory study in which growth of *L. pneumophila* in tap water was enhanced by individual supplements of potassium, up to 100 ppm, and zinc up to 1 ppm (States *et al.*, 1985). A possible reason for this anomaly is that in the current study the dissolved concentrations of metals were measured, whereas States *et al.* (1985) recorded total metal concentrations in the supplements. In addition, differences in the effects of these metals on growth of legionellae may be a reflection of the more complex physical and chemical environments present in the cooling tower.

Iron concentration did not correlate with legionella counts in the present investigation nor in the above field study (Kusnetsov *et al.*, 1993). However, a study investigating

the growth of *L. pneumophila* in hot water tanks found that of 23 chemical parameters measured only iron concentration correlated with the legionella count and that levels of up to 50 ppm (0.9 mM) would enhance growth (States *et al.*, 1985). It was unfortunate that lower levels of iron detection were not available in Modes 1 and 2 as this may have provided interesting data; iron concentration during these modes was less than 0.1 ppm (1.8 μM). However, legionellae and other heterotrophs were isolated from the system even when iron was below the detection limit of the more sensitive electrothermal furnace atomic absorption spectroscopy technique (< 5 ppb or < 0.09 μM). In this sort of study it is not possible to determine the concentrations of nutrients in the growth medium, the cooling water in the tower, independent of the activity of the microflora.

The present investigation found a negative correlation between the total hardness of the water and the concentration of *L. pneumophila*. This is perhaps not surprising since this value is calculated from the concentration of calcium and magnesium measured in the water, but is reported since total hardness is a parameter commonly encountered in water analyses.

In contrast to the results obtained for *L. pneumophila*, the heterotroph population increased with the increasing concentrations of a number of ions, including calcium and potassium, and the hardness of the water. However, the growth of *L. pneumophila* and other heterotrophs appeared to be adversely affected by increased concentrations of zinc.

4.6.6 "Dirtiness"

Various parameters which relate to the level of nutrient in cooling system water have been found to negatively correlate with concentrations of legionellae. The current study found an inverse relationship between the concentration of *L. pneumophila* and conductivity. Kusnetsov *et al.* (1993) reported that the chemical oxygen demand

(COD) of cooling tower water negatively correlated with the concentration of legionellae in the water. Chemical oxygen demand is a measure of the concentration of organic matter, this led these authors to suggest that legionellae favoured waters of low nutrient content. Furthermore, Witherell *et al.* (1986) found that the turbidity of cooling system water was significantly lower in towers which were colonised with legionellae than those from which no legionellae were recovered.

It would appear from the results of the current investigation and the other studies detailed above that systems which are relatively "clean" are more favourable to the multiplication of legionellae than "dirty" systems. This is an interesting proposition and may in part be due to other heterotrophs out-competing legionellae in systems with relatively high nutrient content. Indeed, Kusnetsov *et al.* (1993) found that the total bacterial count was negatively correlated with the legionella count but positively correlated with nutrient concentration. Total bacterial count correlated positively with conductivity in both the current study and in the investigation carried out by Kusnetsov *et al.* (1993). It is possible that in warmer systems legionellae may be able to compete more favourably with other species which have a lower growth temperature optima, a scenario which would add further complexity to any apparent correlations.

4.6.7 Other bacteria

A number of field studies found no significant correlation between total viable counts of bacteria and legionella counts (Broadbent *et al.*, 1992; Witherell *et al.*, 1986; Yamamoto *et al.*, 1992). A significant negative correlation has been reported between culturable legionellae and total bacterial counts as determined by epifluorescence microscopy of acridine orange stained samples (Kusnetsov *et al.*, 1993). Using microscopic counts of stained bacteria and antibody labelled legionellae Pope and Dziewulski (1992) found a positive correlation ($r = 0.770$) between total bacteria and legionella counts. A weak negative correlation between the heterotrophic plate count (HPC) and the legionella plate count was determined in this present study. This may

indicate that different conditions favour the growth of *L. pneumophila* than those which stimulate the growth of the other heterotrophs in the cooling tower.

4.6.8 Other microbes

Yamamoto *et al.* (1992) found significant positive correlations between the numbers of amoebae, ciliates and flagellates and concentrations of legionellae. Other field studies have failed to detect any protozoa in the cooling towers (Kusnetsov *et al.*, 1993; Pope & Dziewulski, 1992). Although Bentham *et al.* (1993b) found amoebae in all the cooling towers they sampled, statistical analysis of the data did not indicate any correlation between legionella concentration and the concentration or species of amoebae present.

4.7 INTERPRETATION OF RELATIONSHIPS

Apparent relationships between concentrations of legionellae and other system parameters should be considered with some caution as the data collected during Mode 1 may contribute disproportionately to the values of correlation coefficients obtained, since it was the only modality to result in heavy colonisation of the system with *L. pneumophila.* Mindful of this fact additional repeats of the *Spring/Autumn* mode were undertaken at the number of concentration cycles (2.5) used through most of the other modes in an attempt to achieve large populations of *L. pneumophila* within the system. The number of cycles of concentrations was selected on the advice of water treatment specialists as being the most suitable, and so the most realistic, for this cooling tower and the chemistry of the water in the make-up supply. It was with the desire to accurately model real cooling tower operation as closely as possible that this level of TDS was adhered to as the control value.

The correlation coefficient calculated for the relationship between *L. pneumophila* and conductivity was -0.5006, which indicates that conductivity accounts for around 25% of the variation in *L. pneumophila* population size. Similarly the correlation between *L.*

pneumophila and the heat exchanger temperature accounts for 15% of the variation in the numbers of legionellae in the system. This is similar to other studies where correlations between numbers of legionellae and temperature have been reported. In the work of Broadbent *et al.* (1992) and Yamamoto *et al.* (1992) water temperature respectively accounted for around 20% and 10% of the variation in the concentration of legionellae recovered.

It is important to note that the range of values measured for some parameters, *e.g.* pH, in this study were probably insufficient for correlations to be apparent.

When reviewing the data presented in studies investigating the factors which affect the growth of legionellae in cooling tower it is evident that there is little or no consensus as to which factors influence the growth of legionellae and that for the most part the correlations which were reported only accounted for a small part of the variation in legionella population size. There are two notable exceptions to this, the first being the correlation between total bacterial count and legionella count reported by Pope and Dziewulski (1992), based on counts of stained bacteria and immunolabelled legionellae, which accounts for 59% of the variation in the legionella count. The second comes from a report by States *et al.* (1987) and concerns a negative correlation between the multiplication of *L. pneumophila* and alkalinity and pH of condenser water samples inoculated with *L. pneumophila* which accounted respectively for 65% and 53% of the variability in the concentration of *L. pneumophila*. It should be noted that these correlations were reduced when data for basin water samples were combined with those for condenser water samples, and that this experiment was carried out in the laboratory and not in a cooling tower.

Correlations between parameters do not necessarily indicate causal associations, and may instead be evidence of some other underlying relationships.

4.8 ASSOCIATIONS BETWEEN LEGIONELLAE AND OTHER WATER BACTERIA

There have been a number of reports of heterotrophic bacteria either stimulating or inhibiting the growth of *Legionella* species. Toze *et al.* (1990) reported that 32% of heterotrophic plate count bacteria isolated from chlorinated drinking water inhibited the growth of *Legionella* species, with 16% inhibiting the growth of *L. pneumophila*. They also demonstrated the ability of two HPC isolates (which they were unable to identify) to both inhibit and enhance the growth of a number of *Legionella* species depending on the composition of growth medium. *Aeromonas* species and a strain of *Pseudomonas vesicularis* inhibited all the *Legionella* species tested including *L. pneumophila*. In the current study one of the HPC isolates was identified as being *P. vesicularis* and another as an *Aeromonas* species. It is interesting to consider the possibility that these bacteria inhibited growth of *L. pneumophila* in the cooling tower. In further studies Toze *et al.* (1994) found that 32 out of 33 strains of *Aeromonas* tested were inhibitory to *L. pneumophila*. Inhibition of *Legionella* species by other water heterotrophs has been attributed to competition (Kusnetsov *et al.*, 1993) and a diffusible bactericidal compound (Toze *et al.*, 1993). Legionella growth supporting activity has been reported for a number of water bacteria: *Acinetobacter, Alcaligenes, Flavobacterium and Pseudomonas* (Stout *et al.*, 1985); *Flavobacterium breve* (Wadowsky & Yee, 1983); and pigmented Gram-negative nonfermentative bacteria (Wadowsky & Yee, 1985). Growth supporting activity has been attributed to the provision of L-cysteine, for which legionellae are auxotrophic, by synergistic bacteria (Wadowsky & Yee, 1983, 1985). This is an area which will receive further investigation in future studies with the experimental cooling tower.

A characteristic of many heterotrophic bacteria in treated drinking water is the ability to form brightly coloured non-photosynthetic, non-diffusible pigments (Reasoner & Geldreich, 1985). A number of the isolates routinely recovered from the cooling tower

formed highly coloured colonies on agar, being yellow, orange or pink. The concentration of the orange isolate from the cooling tower showed a significant negative relationship with *L. pneumophila* density. This isolate was only present during Modes 2 and 3 which were part of the same experimental run, therefore, its occurrence may have been due to some change in the composition of the heterotroph population of the make-up water during this period. Interestingly, the converse effect, legionella growth enhancement, by an isolate similar to the orange pigmented bacteria in this study has been reported (Wadowsky & Yee, 1985). The isolates were not successfully identified in either study and so may not be the same species.

A common motif to the above investigations, in addition to the ubiquity of bacteria in water which will either support or inhibit the growth of *Legionella* species, is the degree of difficulty and uncertainty which the various authors ascribe to the task of identifying the non-legionella water isolates (Wadowsky & Yee, 1983; Stout *et al.*, 1985; Toze *et al.*, 1990). Identification of water isolates particularly to the species level requires caution as water contains a wide variety of bacteria, many of which are poorly described and recalcitrant to identification by existing methodologies (Reasoner & Geldreich, 1985; Ward *et al.*, 1986).

4.9 SURFACE ASSOCIATED GROWTH

Legionella pneumophila was isolated from biofilms which grew on a variety of materials (glass, brass, stainless steel and galvanised steel) immersed in the pond of the cooling tower at concentrations comparable to those reported in other studies (Schofield & Wright, 1984; Bezanson *et al.*, 1992). It was not appropriate to investigate the relationship between the number of legionellae growing in biofilms with their concentration in the bulk water phase due to the paucity of biofilm data from the early experimental runs. The biofilm concentrations of legionellae in the current study were 100-fold lower than those reported by Rogers *et al.* (1994a). This is probably due to

differences in the design of the experimental systems as well as differences in water chemistry, pH, dissolved oxygen tension and other physicochemical parameters, and the nature of the substrata used to culture biofilms. In the two-stage chemostat model used by Rogers *et al.* (1994a) the vessel in which biofilms were generated was continuously seeded with legionellae from the first vessel; the cooling tower was inoculated once and in the absence of any legionellae in the make-up water did not receive subsequent inocula. The cooling tower was simulating a single event contamination which may occur during construction work or during damage or repair to a water system. A number of outbreaks of Legionnaires' disease have been associated with such perturbations to water or cooling systems (Marks *et al.*, 1979; Storch *et al.*, 1979; Morton *et al.*, 1986; Mermel *et al.*, 1995). Both these models are valid but were designed to address different questions. The two-stage chemostat model (Rogers *et al.*, 1994a) was suited to comparing the ability of plumbing materials to become contaminated with legionellae under defined conditions. The cooling tower was developed to mimic the growth environment in evaporative cooling systems and to determine its influence on the growth of populations of legionellae in relation to operational practice.

Although regular biofilm samples were obtained later in the course of the study, *i.e.* during Modes R1 and R2, no legionellae were isolated from these samples. The *L. pneumophila* counts obtained from water samples were significantly lower during these later experimental runs. Concentrations of legionellae isolated from planktonic samples collected at the same time as biofilm samples were between zero and 10^4 CFU l^{-1}, with 200 to 300 CFU l^{-1} being the planktonic concentration during most of the mode, which is considerably lower than during Mode 1. Wireman *et al.* (1993) reported that legionellae were not detected in biofilm samples on galvanised steel when the planktonic concentration was 10^6 l^{-1}. Thus the lack of legionellae in biofilm samples collected in the present study may have been attributable to their low concentration in the recirculating water. In addition a number of reports indicate that recovery of

legionellae from biofilm samples can be intermittent (Wright *et al.*, 1989; Bezanson *et al.*, 1992; Bentham *et al.*, 1993b).

Total heterotroph counts from biofilm samples were up to 10^9 CFU m^{-2} which is comparable with other studies (Rogers *et al.*, 1994a, b). Given the low concentration of *L. pneumophila* in the cooling water and the relatively small surface area which it was practical to sample it is feasible that *L. pneumophila* was present in biofilms within the system but the sampling method was not sufficiently sensitive to detect it. Fluctuations in the number of legionellae isolated during Modes R1 and 3 may have been due to biofilm sloughing off parts of the system at intervals leading to increased planktonic concentrations. This is supported by the conclusions of a field study which suggested that the heat exchanger is the primary focus of legionella growth and that sessile populations of legionellae in this part of the system seed the rest of the cooling tower (Broadbent *et al.*, 1992; Bentham *et al.*, 1993a, b).

4.10 DISINFECTION

The cooling tower was disinfected according to Recommendation 7 of the DoH Expert Advisory Committee on Biocides (Department of Health, 1989) *i.e.* "Emergency cleaning should always take place if a system is implicated or suspected of being implicated in an outbreak or large numbers of legionellae are found ... If chlorine is used (as a biocide), however, a level of 50 mg l^{-1} (ppm) of free available chlorine should be maintained for at least 4 hours." This was found to reduce the concentration of *L. pneumophila* in the water from 465,000 CFU l^{-1} to below the level of detection (100 CFU l^{-1}). However, *L. pneumophila* was isolated from the heat exchanger sediment after hyperchlorination. Although the water temperature and pH during the trial were not optimal for chlorine disinfection they were representative of conditions which are likely to occur in the field. The protocol was strictly adhered to with the level of free available chlorine being monitored throughout the period and samples

collected 4 hours 50 minutes after initial dosing.

It should be noted that although this protocol is the most rigorous of all those recommended for the disinfection of problem cooling water systems it failed to eradicate *L. pneumophila* from the cooling tower.

The finding that hyperchlorination does not eradicate legionellae from a cooling tower system is consistent with the findings of an Australian field study (Broadbent *et al.*, 1992) and with the observation that under certain circumstances shock chlorination at $\geq$ 50 ppm does not effectively control the growth of legionellae (Muraca *et al.*, 1988). The results of this study support the requirement for increased attention to be given to the heat-exchanger as suggested by Bentham *et al.* (1993a) as this part of the system may act as a reservoir of legionellae even after decontamination procedures have been carried out.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1 CONCLUSIONS

The results presented in this thesis clearly demonstrate that the growth environment has a profound influence on the growth, physiology and virulence of *Legionella pneumophila*. Growth temperature was shown to modulate reversibly the virulence of this bacterium; with temperatures similar to those encountered in evaporative cooling towers resulting in increased virulence. The bacterium also modified its physiology in response to changes in the growth temperature, including growth efficency, modifications in the cellular composition in order to maintain membrane fluidity, and the accumulation of a carbon storage compound. *Legionella pneumophila* was also influenced by the availability of iron; under iron-limited conditions it expressed siderophore-like activity and altered metabolism. Surprisingly, iron-limited cultures of *L. pneumophila* were less virulent than iron-replete cultures. This may be due to a change in the iron-content of the bacterium prior to infection.

This study also supported earlier work on the physiology of legionellae and added new data to this area including evidence of the metabolic versatility and the microaerophilic nature of these organisms. This study provided the first direct evidence that *L. pneumophila* accumulates the carbon and energy storage polymer, polyhydroxybutyrate (PHB). Furthermore, data collected in this study suggested that metabolism of PHB may support the growth of legionellae in oligotrophic environments. In addition, a biochemical method for the identification of legionellae was developed.

Temperature was found to significantly enhance the growth of *L. pneumophila* in a cooling tower; conversely, conductivity demonstrated a negative correlation with the concentration of *L. pneumophila* in the system. In common with a number of previous studies no strong correlation between *Legionella* and total bacterial counts was found,

and given the disparity in relationships reported between these two parameters it would seem unadvisable to relate the total bacterial loading of a system with its likelihood to contain legionellae.

The findings of this study would suggest that the rationale upon which control strategies are based should not only seek to minimise the chance of legionellae encountering conditions which may lead to amplification of numbers but also to minimise exposure to conditions which may enhance its virulence.

5.2 FUTURE WORK

The defined medium chemostat culture system could be used to investigate the influence of other environmental parameters or combinations of these on the physiology and virulence of *L. pneumophila*.

The study of the effects of iron-limitation on *L. pneumophila* was continued in association with a colleague and the extension to the work presented herein forms the basis of another research project in which the mechanisms by which *L. pneumophila* obtains iron *in vivo* were investigated.

Additional work should be carried out to further elucidate the role of PHB in the survival of legionellae in oligotrophic or other stressful environments.

Given adequate time and resources it should be possible to create a stable database for the modified BIOLOG system to enable the biochemical identification of legionellae which would be a useful addition to existing techniques.

The microbiologically-contained cooling tower is a unique facility and there is considerable scope to build upon the research presented in this thesis. Future studies

should include the following topics:

Further investigation of the inverse relationship between conductivity and the concentration of legionellae. This could be undertaken by operating the cooling tower at cycles of concentration (*i.e.* concentrations of dissolved solids; see Appendix) both higher and lower than those usually recommended. This would confirm the observation and determine the range of conductivity for which it is valid.

Recent modifications to the system allow the cooling tower to be operated at higher temperatures, thus facilitating further experiments to elucidate the influence of temperature on the growth of legionellae in cooling towers. It is now possible to operate the tower with heat exchanger temperatures which encompass the operating temperature range of the majority of evaporative cooling applications.

The influence of water chemistry could be investigated by artificially modifying the make-up water, by for instance softening the water supplied to the tower. Alternatively, water collected from different sources could be used, although this could present logistic problems.

An extended investigation of the role of biofilm growth in the survival and multiplication of *L. pneumophila* in the cooling tower would be of considerable value, and may yield data which would be important in the development of modified control strategies.

An aerosol sampling point was incorporated into the exit duct of the cooling tower on the "dirty" side of the HEPA filters. Utilising this sampling point it will be possible to investigate the relationship between the concentration of *L. pneumophila* in the recirculating water and in the exit air stream. This would provide data from which a rational basis for determining a "safe-level" of legionellae in cooling water could be

derived.

Having established operational conditions which result in low, intermediate and high concentrations of *L. pneumophila* in the cooling tower, fuller studies on the ecology of the system can be undertaken. This would involve a study focussing upon the role of amoebae and protozoa in the ecosystem and would be undertaken in association with experiments aimed at elucidating the interactions between these microbes, other bacteria and legionellae.

The microbiologically-contained cooling tower offers a well defined and reproducible environment which accurately models operational evaporative cooling towers, and thus is ideally suited to the study of methods for control of legionellae. Utilising this system it is possible to carry out pseudo-field trials of biocides against high concentrations of *L. pneumophila* safely. Similar studies can also be carried out with non-chemical control methods such as ultraviolet irradiation and side-stream pasteurisation.

It may be possible to repeat the chemostat experiments which demonstrated temperature modulation of virulence using inocula recovered from the cooling tower under a variety of operating conditions. Thus it may be possible to establish if growth in evaporative cooling towers can modify the virulence of legionellae, and if so, which operational conditions lead to this event. Clearly such information would be valuable in further developing a scientific basis for control of the spread of Legionnaires' disease from evaporative cooling towers.

APPENDIX

MAIN COMPONENTS OF AN EVAPORATIVE COOLING TOWER (SEE FIGURE A.1)

The case or shell. The structure enclosing the heat transfer process and acting as a platform for the other components.

The fan. Air movement through the tower is facilitated by the fan which can be positioned to either pull (induced draught) or push (forced draught) air through the tower.

Drift eliminators. Drift eliminators are positioned in the path of the outlet air stream to prevent water droplets being carried out of the tower, *i.e.* to minimise drift loss.

Water distribution system. Water must be spread evenly across the cross-section of the tower, two methods commonly used in mechanical draught towers are trough and gutter or spray distribution water systems.

Packing/pack. The packing of the cooling tower consists of a system of baffles which break the water up into small droplets and slow its progress down through the tower to the pond thus maximising contact between water and air by increasing surface area and reducing film thickness.

Pond/cold water basin. The pond is the area where cooled water is collected before recirculation to process cooling.

TERMINOLOGY OF COOLING TOWER OPERATION

Make-up. Water added to the circulating water system to replace leakage, evaporation, drift loss and purge.

Purge. Water deliberately discharged from the system in order to reduce the concentration of impurities in the circulating water.

Drift loss. Water loss caused by aerosolised droplets carried from the cooling tower by the outlet air (it is drift which has the potential to carry legionellae to susceptible individuals).

Concentration ratio/concentration cycles. The ratio of the total mass of impurities in the circulating water to the corresponding mass in the make-up water.

Heat/Cooling load. Rate of heat removal from the water flowing through the tower expressed in kilowatts.

Hot water temperature. Temperature of water entering the distribution system (also referred to as on-temperature).

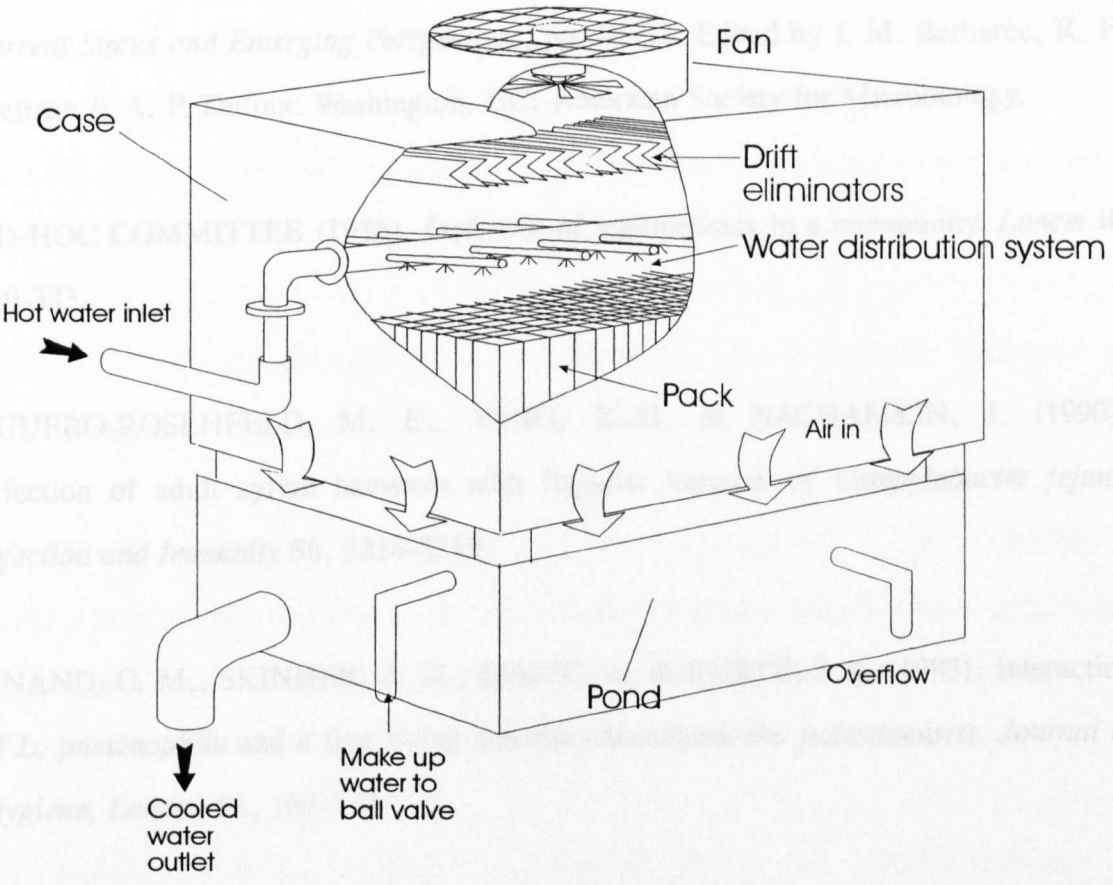
Re-cooled water temperature. Average temperature of water at the discharge point from the pond excluding the effects of the make-up.

Cooling range. The difference between hot water temperature and the re-cooled water temperature.

Approach. The difference between the re-cooled water temperature and the inlet wet bulb temperature.

Wet bulb temperature. Temperature measured by a wet bulb thermometer, usually situated at the air inlet of the cooling tower.

Figure A.1 Diagram of an induced draught evaporative cooling tower.



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